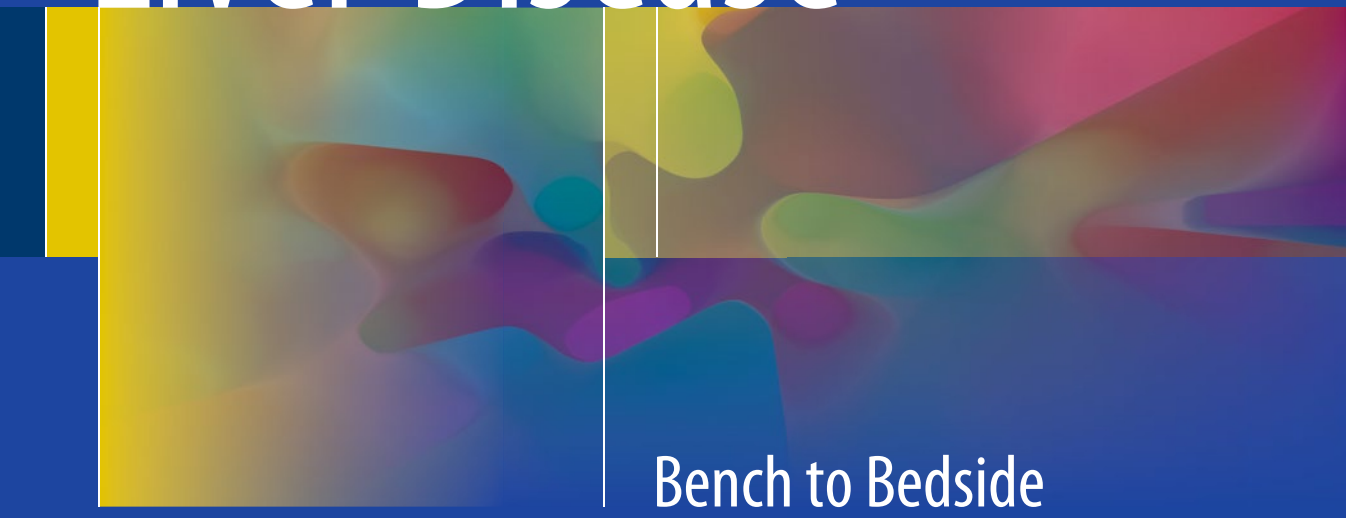
Alcoholic and Non-Alcoholic Fatty Liver Disease



Bench to Bedside

Naga Chalasani
Gyongyi Szabo
Editors

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To my parents, Sujani and C.N. Rao, for their big dreams and sacrifices, and to my brother, Prasad, who is always there for me; To my wife, Satya, and to our son, Sai, who are the joy of my life; To my namesake, DWC, who is behind most of my major professional milestones.

Naga Chalasani

To my family, mentors, and friends.

Gyongyi Szabo

Preface

Alcoholic liver disease (ALD) and Nonalcoholic fatty liver disease (NAFLD) are among the most common causes of cirrhosis, liver failure, and liver cancer worldwide. Histologically, they resemble each other and they both share several common pathogenetic mechanisms. Over the last decade, steady progress has been made in almost all aspects of these two diseases, and this book reflects our attempt to integrate these new developments into existing disease pathogenesis and management paradigms. This book is developed as a state-of-the-art resource for medical students, clinicians, pathologists, clinical researchers, basic science investigators, and pharmaceutical industry worldwide. At the outset, we believed that a single book that includes both ALD and NAFLD would be highly desirable for various stakeholders, but organizing various chapters into a logical sequence turned out to be somewhat challenging. After much deliberation, we have decided to divide our book into multiple sections (e.g., epidemiology, pathogenesis, and clinical features) and then have both ALD and NAFLD chapters next to each other to make it easier for the readers. All contributors, who are experts in their respective areas, have done a masterful job with their chapters and we are indebted for their contributions.

Drs. Mariana Lazo and Mack Mitchell start the book with their review of the epidemiology and risk factors of ALD, followed by a detailed review of the epidemiology and risk factors of NAFLD by Drs. Zobair Younossi and Abhijit Chowdhury. These two chapters set the stage for two marvelous chapters on the pathogenesis of ALD (Drs. Gavin Arteel and David Crabb) and NAFLD (Dr. Jacquelyn Maher). Drs. Jun Xu and Hide Tsukamoto provide an outstanding review of various animal models for studying ALD, and Drs. Mariana Machado and Anna Mae Diehl have done an equally elegant job in describing a large number of animal models which have been developed in the recent past to investigate NAFLD. There have been several major developments in better understanding the genetic basis of these diseases (especially NAFLD), and Drs. Silvia Sookoian and Carlos Pirola have provided an in-depth review of the genetic basis of both ALD and NAFLD in a single chapter.

Moving to more clinical chapters, Dr. Craig McClain and colleagues review clinical features, disease modifiers, and natural history of ALD and alcoholic hepatitis, whereas Drs. Dawn Torres and Stephen Harrison describe in detail the clinical features, disease modifiers, and natural history of NAFLD. Currently, there are no approved therapies for either alcoholic hepatitis or nonalcoholic steatohepatitis (NASH), but there is tremendous interest among various stakeholders to develop novel and effective treatments

for these conditions. To conduct registration clinical trials that are needed for regulatory approval, we must have valid diagnostic criteria and therapeutic end points. The landscape in this area is rapidly evolving and Drs. Cheong, Stein, and Bataller describe state-of-the-art diagnostic criteria for various sub-phenotypes of ALD and therapeutic end points for various stages of clinical trials in individuals with various forms of ALD. The clinical trials' landscape for NAFLD and NASH has dramatically changed over the last 3 years, and there is an evolving consensus on "NASH without fibrosis" and "NASH with advanced fibrosis" as approvable indications from a regulatory standpoint. Also, clear-cut guidance from the regulatory agencies is becoming available with regard to primary end points for Phase 2 and Phase 3 clinical trials in NAFLD. Dr. Arun Sanyal, a pioneer in the field of NASH clinical trials, describes valid diagnostic approaches and clinical end points for treatment in NAFLD and NASH in Chap. 11.

Liver histology remains the gold standard for characterizing the severity of NAFLD and for diagnosing NASH, and there are recent studies showing the prognostic value of liver histology in individuals with alcoholic hepatitis. Drs. David Kleiner and Pierre Bedossa, two expert hepatopathologists, tackle the liver histology in ALD and NAFLD in a comprehensive fashion. NAFLD and ALD are strongly associated with hepatic and extrahepatic malignancies, and Drs. Vasilis Vasilou and Sam Zakhari thoroughly cover hepatic and extrahepatic malignancies in ALD, whereas Drs. Fabio Nascimbeni and Vlad Ratziu describe the putative mechanisms and risk factors of malignancies in NAFLD with a special emphasis on the risk of hepatocellular carcinoma in individuals with non-cirrhotic NAFLD.

Alcoholic hepatitis continues to carry significant morbidity and mortality and unfortunately no new effective therapies have been developed for this condition in nearly 3 decades. The role and timing of liver transplantation for individuals with acute alcoholic hepatitis remain controversial and geographically variable. Dr. Szabo and Dr. Jan Petrasek comprehensively describe existing and emerging therapies, novel targets, and the role of liver transplantation for individuals with various forms of ALD. As stated before, there is tremendous interest in developing effective pharmacological agents for NASH and there has been an explosion of Phase 2 and Phase 3 trials in this space. Drs. Samer Gawrieh and Naga Chalasani review various treatments including lifestyle modification, bariatric surgery, and new compounds to treat liver disease and coexisting comorbidities in individuals with NAFLD. Last but not the least, Drs. Hannah Awai and Jeffrey Schwimmer provide a comprehensive review of NAFLD in children, a growing problem with substantial disease burden.

As research is progressing rapidly in these two disorders, we suspect some important articles that have just been published may be missing in this book. Regrettably, such omissions are unavoidable due to lengthy editing and publishing process involved in developing a multiauthored textbook. Nonetheless, we hope this book provides an updated, broad, and practical review of all aspects of ALD and NAFLD.

Indianapolis, IN — Naga Chalasani
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Acknowledgments

We are indebted to all contributing authors as this book would have not been possible without their fantastic chapters. Despite their overwhelming responsibilities and competing demands, they graciously agreed to take yet another commitment and delivered their work in a timely fashion. There are important developments in the field almost on a daily basis and such developments are possible because of funding agencies across the world investing in biomedical research, emerging and established scientists and investigators continuing to pursue high-quality research, pharmaceutical industry exploring new therapeutic frontiers, and importantly, patients worldwide offering themselves through their participation in human investigations and early and late phase clinical trials. We are very thankful to all these stakeholders. We offer our sincere thanks to the publisher and to Ms. Tracy Marton who has done an outstanding job in developing this book.

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Epidemiology and Risk Factors for Alcoholic Liver Disease

1

Mariana Lazo and Mack C. Mitchell

Epidemiological studies are the foundation for disease control and prevention. Some epidemiological studies track the occurrence of the disease (e.g., prevalence and incidence) in the population, while others contrast different populations and attempt to explain the causes of the disease at the population level. Typical study designs addressing these types of questions are cross-sectional and ecological studies. Cohort studies and well-conducted case-control studies are ideally suited to identify individual risk factors for a disease. The Dionysos study, a population-based study of the relationship between alcohol consumption and liver disease, is a prototype of this type of study. Rigorous cohort studies are also instrumental for characterizing the incidence of the disease and its natural history. Validity and reliability studies embedded into larger epidemiological investigations provide additional valuable information regarding the accuracy of diagnostic methods, whereas randomized clinical trials are particularly important for testing the effects of therapeutic modalities. Recently developed reporting guidelines, such as CONSORT (for clinical trials) [1] and STROBE (for observational studies) [2], are excellent resources for users of medical literature as guidelines for systematically evaluating the quality of the study.

This chapter will provide a summary of the most important epidemiologic evidence on alcoholic liver disease (ALD) throughout the world. Risk factors identified from population-based and case-control studies will be discussed. Other important aspects of ALD such as natural history and treatment are thoroughly addressed in other chapters of the book. In the context of ALD, a number of key studies have used the publicly available mortality and alcohol consumption data from the World Health Organization (WHO) to demonstrate the strong correlation between per capita alcohol consumption and cirrhosis mortality. National surveys provide a constant resource of data at the national level that can be useful in comparing countries with differences in per capita consumption and patterns of alcohol consumption as well as longitudinal changes over time.

For any epidemiological study of ALD, two fundamental issues must be addressed in the interpretation: (1) measurement of alcohol intake and other variables such as body mass index, hepatitis C infection, etc., that may impact the presence of ALD and (2) definition of the presence of ALD including the onset and stage of the disease.

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Estimates of the Risk Attributed to Alcohol

Given that the evidence supporting the association between alcohol and liver disease has been considered truly causal in nature, two measures of association that refer to the proportion of the risk that is attributed to the exposure have been estimated: (1) attributable fraction *among the exposed* and (2) *population*-attributable fraction. Broadly speaking, these measures are used to indicate the impact of a given exposure or the proportion of occurrences that may be reduced if the exposure were to be eliminated.

Attributable risk among the exposed, often expressed as a percentage, indicates the proportion of the risk of a given outcome among the exposed group that is attributable to the exposure of interest [3]. It is based on estimates of incidence among the exposed and unexposed from cohort studies. Although it seems that it would have very practical implications, epidemiologists remain somewhat skeptical and cautious with the use of this measure given that the assumption of a causal relationship without confounding is a very strong assumption.

Population-attributable fraction (PAF) is related to the previous measure but with significant appeal to public health workers and policy makers because of its applicability to the entire population. PAF is supposed to represent the proportional reduction in the average disease risk in the *population* that would be achieved by eliminating a given exposure from the *population* while assuming that the rest of the risk factors remain unchanged [3]. Its calculation includes both the *unbiased* estimate of the association between the exposure and the outcome and the prevalence of the exposure at the population. Like the previous concept, it relies on very strong assumptions with respect to the causal link between the exposure and the outcome and with respect to the estimate of risk. In spite of the limitations, this measure may assist policy makers in prioritizing and estimating the impact of selected interventions.

For estimates of the risk associated with the exposure, investigators often rely on meta-analyses that pool data from several studies. Although these meta-analyses increase the precision of the estimates, the quality of individual studies impacts the validity of the results and therefore may still lead to biased estimates.

Spectrum of Alcoholic Liver Disease

Three overlapping conditions have been found in individuals who drink excessively. Alcoholic steatosis or fatty liver is a reversible condition with a good prognosis if the affected individual remains abstinent from alcohol. Alcoholic steatohepatitis is a more severe type of injury characterized by ballooning degeneration, neutrophilic infiltration, and the presence of Mallory–Denk bodies. There is usually active pericellular fibrosis in these individuals and the condition may progress to cirrhosis even in those who remain abstinent from alcohol. Those with cirrhosis due to alcohol may or may not have associated active alcoholic hepatitis. Fat is usually not prominent in those with cirrhosis. The risk factors for alcoholic hepatitis and cirrhosis may differ from those for simple steatosis.

At a clinical level, individuals with evidence of liver damage and a history of excessive and prolonged alcohol consumption are usually considered to have ALD if no other obvious cause such as chronic viral hepatitis is identified. However, this definition, focused on a single risk factor, may be problematic as it is possible that more than two of these causes are present. In fact, as briefly discussed later on this chapter, there is evidence suggesting that those individuals with excessive alcohol consumption and viral hepatitis or obesity are at much higher risk of liver disease that those with a single risk factor. These observations provide a conceptual basis to move from a model of a specific disease, namely, ALD, toward a model of postulated causal risk factor(s) contributing to the development of a particular outcome/stage (steatosis, alcoholic hepatitis, or cirrhosis) or complications such as mortality associated with liver disease (Fig. 1.1). Some epidemiological studies focus on complications of the disease process such as mortality that

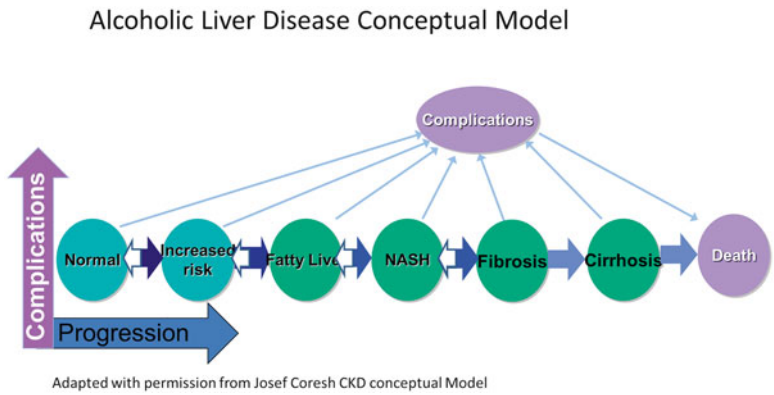

Fig. 1.1 Conceptual model. As alcoholic liver disease progresses from fatty liver to cirrhosis, the risk of complications increases. Death related to these complications can develop at any stage of the disease but is more likely in the advanced stages. Thus mortality related to ALD does not always imply the presence of cirrhosis. Alcohol can be viewed conceptually as a risk factor for progression of disease as well as a cause of liver disease

theoretically could occur at any stage of the disease. Although the probability of an outcome such as mortality varies according to the stage of disease, an outcome such as mortality does not necessarily indicate a particular stage of the disease. Mortality can occur at the stage of alcoholic hepatitis or cirrhosis or rarely even at the stage of fatty liver alone.

A major barrier for the characterization and study of ALD is the limited set of biomarkers or other diagnostic criteria for different stages of the disease other than liver biopsy. Simply relying on biopsy is not feasible most of the times, in particular in epidemiological studies. Recently developed noninvasive methods (i.e., ultrasound or MR elastography) are providing encouraging results; however, more research is needed to fully characterize their use as diagnostic criteria for stages of ALD.

Worldwide Burden of Alcoholic Liver Disease

In 2010, the Global Burden of Disease Study estimated that alcohol-attributable cirrhosis was responsible for 493,300 deaths in the world (roughly 157,000 among women and 336,400 among men), representing 0.9 % of all the deaths. In addition, it estimated that alcohol accounted for 48 % of all deaths due to cirrhosis. The estimated number of deaths from alcohol-attributable liver cancer was 80,600, with a strikingly higher burden among men (65,900 deaths) than among women (14,800) [4] These estimates have been widely used by the WHO for the preparation of the Global Status Report on Alcohol and Health 2014 [5] and for their emphatic recommendation of interventions to reduce alcohol consumption as a priority for public health agencies. This recommendation was based on their conclusion that even small amounts of alcohol may contribute to development of liver disease or deaths due to liver disease in a vulnerable host. However, the conclusion has been challenged on the basis that consumption of low to moderate amounts of alcohol may have different effects, including potential benefits, than those observed in heavier drinkers.

In the next few paragraphs, we will discuss the main sources of data for that type of studies and the methods used to obtain these estimates.

Mortality Statistics

Figure 1.2 shows the cirrhosis-related mortality rates in several countries by sex in 2000 and 2010, illustrating the trends over time and geographical variations (Fig. 1.2). Similar data have

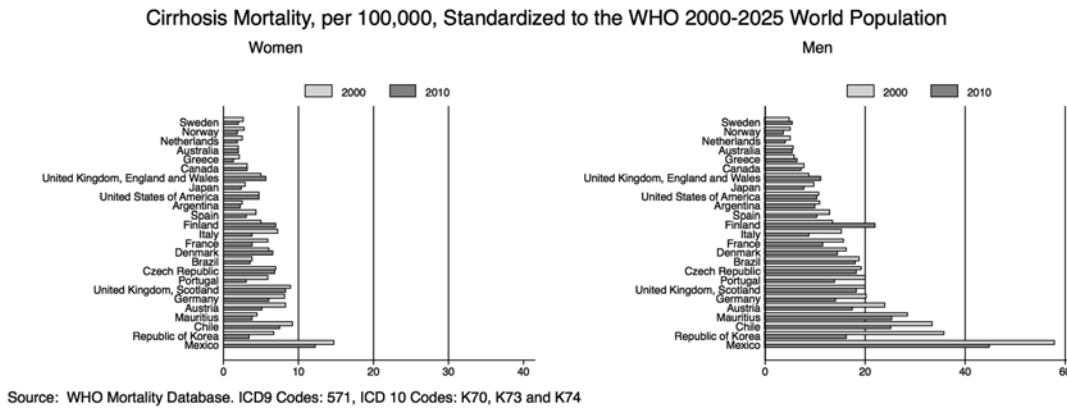

Fig. 1.2 Trends in cirrhosis mortality 2000–2010 in selected countries by sex. Cirrhosis mortality rates among different countries vary considerably. In some countries, the mortality rate decreased between 2000 and 2010, whereas in others such as the UK and Finland, the rate increased

been reported elsewhere [4, 6]. While these data are widely used by public health agencies to define the burden of ALD in the population, it is very important to realize that the mortality statistics underestimate the true burden of the disease. The use of the international classification of disease (ICD) has attempted to standardize the completion of death certificates throughout the world. Although this effort has been very important, changes in the ICD classification codes and level of detail have made the evaluation of trends more difficult.

In addition, two important issues must be considered in the interpretation of these types of studies: (1) Clinicians are well aware of the difficulty in establishing cirrhosis as an underlying cause of death. (2) These trends fail to distinguish between alcoholic versus other cause of cirrhosis. For death certificate and other data using ICD codes, the attribution of alcohol versus other causes is highly subjective and may be biased. Studies validating death certificates with data from hospital records and other sources have estimated the number of deaths from cirrhosis with no mention of alcohol reassigned to alcohol related more than doubles [7].

Finally, it is important to recognize that it is highly likely that misclassification in death certificates has significant social, regional, and temporal trends.

National Estimates of Alcohol Consumption

The WHO has established a surveillance system throughout the world to record per capita consumption of ethanol and to estimate the prevalence of lifetime abstainers, former drinkers, and current drinkers (http://www.who.int/gho/alcohol/en/).

These estimates are largely based on representative surveys in each of the countries. While misclassification can be expected to occur more frequently among those with heavy consumption, broadly speaking, self-reported alcohol consumption can be considered a valid measure of the population on average.

Most of these ecological studies have countries as unit of analysis as opposed to individuals. Their inferences are particularly useful to characterize population level determinants of the disease. There is an intrinsic relationship between diseased individuals and the whole population from which they come. In the context of the association between alcohol and liver disease, this observation has a long history. In 1961, Dr. Gerald Klatskin highlighted the strong significant linear correlation between death rate from cirrhosis and the average per capita consumption of alcohol in 1939 [8]. That remarkably high correlation has been replicated over time (Fig. 1.3).

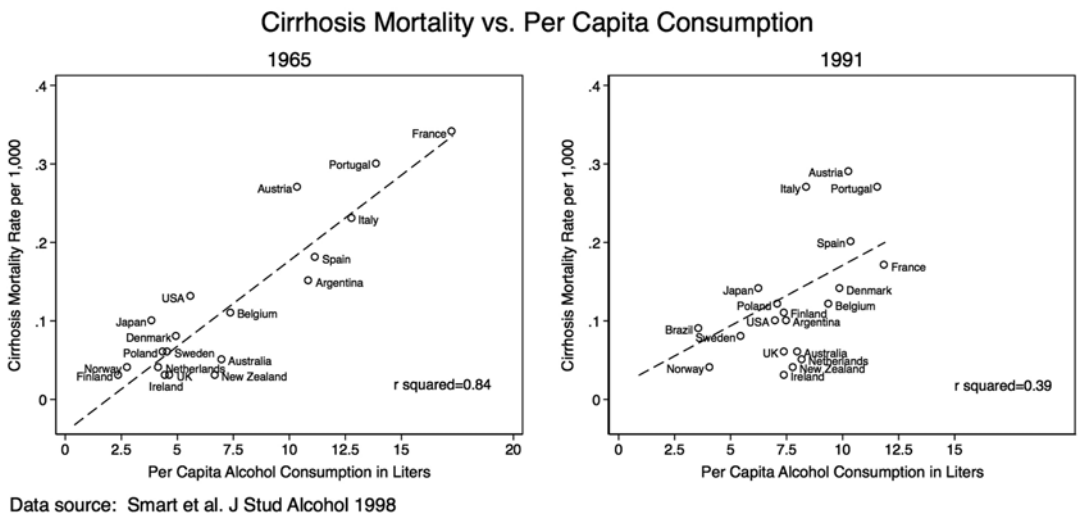

Fig. 1.3 Ecological studies: Relationship between cirrhosis mortality and per capita alcohol consumption, 1965 and 1991. The relationship between per capita alcohol consumption and cirrhosis mortality rates was more linear in 1965 than in 1991. The differences are more likely related to changes in classification of cirrhosis than to estimation of per capita alcohol consumption

Risk Factors for Alcoholic Fatty Liver Disease (AFLD)

Fatty liver is likely a metabolic consequence of ingesting moderately large amounts of ethanol. Studies have demonstrated that alcohol decreases beta oxidation and synthesis of fatty acids in the liver thus increasing the supply of fatty acids [9]. Studies in human volunteers demonstrated unequivocally that feeding 68–130 g of ethanol daily with a diet of 16 % protein and 36 % fat or 25 % protein and 25 % fat resulted in a significant increase in hepatic triglycerides and changes within the ultrastructure of mitochondria in all subjects within 6–14 days [10–12]. Similar changes were observed in volunteers after drinking 270 g of ethanol in addition to a standard diet for only 2 days [10, 11]. None of the volunteers had blood alcohol concentrations (BACs) above 80 mg/dl during the time of the study. These findings suggest that heavy binge drinking rapidly results in fatty infiltration of the liver, even in the absence of a high BAC.

A number of epidemiological studies have demonstrated the strong association between *excessive* alcohol consumption and hepatic steatosis [13]. A case-control autopsy study of individuals who died in automobile car crashes in the USA showed a prevalence of fatty liver (56 %) in those who had a blood alcohol concentration >0.08 % at the time of death and were presumably heavy drinkers based on reports from family members [14]. Many other studies have reported a similar prevalence of fatty liver in alcoholics admitted to detox centers [15, 16]. Selection bias is a common threat to the validity of this type of studies since evaluation of subjects is not performed in a random fashion.

Some of the best US population-based studies that assessed hepatic steatosis include the Dallas Heart Study (DHS) [17] and the Third National and Nutrition Examination Survey (NHANES III) [18]. While in the NHANES III, a significant association between elevated alcohol consumption and hepatic steatosis was observed (Fig. 1.4); the DHS did not demonstrate such association. It is possible that the number of individuals with heavy alcohol consumption in the DHS was not sufficient to examine this association.

Similar studies from Europe examined hepatic steatosis in relationship to both alcohol consumption and obesity. A population-based sample of individuals in Northern Italy, the Dionysos study,

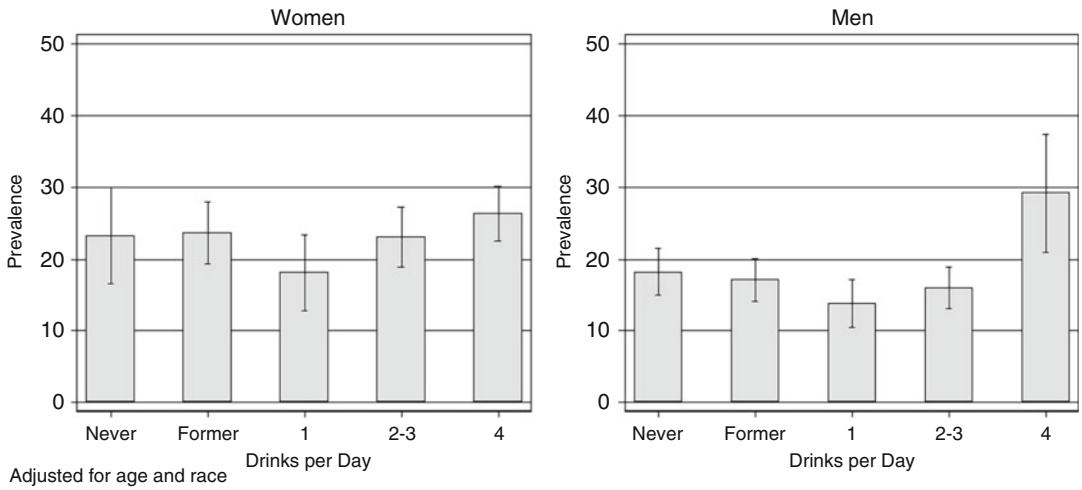

Fig. 1.4 Age- and race-adjusted prevalence of steatosis by usual number of drinks per day. US Adult Population, 1988–1994. (NHANES III). Steatosis in the NHANES III cohort was evaluated using hepatic ultrasound. As noted above, the prevalence of steatosis in men drinking ≥ 4 drinks daily was greater than in never or former drinkers, while the prevalence of steatosis was slightly lower in those drinking one drink/day compared to either never drinkers or those consuming more than three drinks daily. This U-shaped or J-shaped relationship between alcohol consumption and disease has also been observed for coronary heart disease and several other outcomes

estimated that the prevalence of fatty liver determined by ultrasound imaging was 46 % in non-obese, heavy drinkers (>60 g daily intake), 76 % in obese individuals with BMI > 30, and 95 % in obese, heavy drinkers [19]. Another study conducted in France identified obesity as a significant risk factor for steatosis (OR = 2.5, 95 % confidence interval 1.0–6.6), in a sample of patients with heavy alcohol consumption (>50 g daily) even after adjustment for age, sex, duration of alcohol abuse, and amount of alcohol consumption [16]. Of note, both Italy and France are predominantly wine-drinking countries in which the pattern of consumption of alcohol as well as the preferred beverages may differ from North America.

As opposed to the consistent positive association between heavy alcohol consumption and hepatic steatosis, more recent studies suggest a protective effect of low to moderate amounts on the prevalence of hepatic steatosis (Fig. 1.4) [20–22]. One possibility is that low doses of alcohol may exert beneficial effects on insulin resistance and other metabolic parameters that are important in nonalcoholic fatty liver disease (NAFLD). Observational studies have identified a significant body of evidence suggesting that consumption of moderate amounts of alcohol is associated with decreased risk of diabetes [23]. It is therefore plausible that the small improvements in insulin resistance (in particular hepatic insulin resistance) counterbalance the pro-steatotic effect of alcohol [20, 21]. However, the possibility that the epidemiological results are confounded by differences in diet or other aspects of a healthy lifestyle pattern frequently observed among moderate drinkers is difficult to exclude as an explanation.

The interaction between alcohol and other components of the diet in development of fatty liver has been examined in small metabolic studies [10–12, 24]. Even though fat accumulation occurred in almost all subjects who were fed large amounts of ethanol, the extent of fatty liver was greater in those subjects fed a high-fat diet with ethanol. Although a number of large cohort studies have examined the relationship between diet, alcohol, and heart disease, there are relatively few that have reported the relationship between ethanol and fat intake in the development of fatty liver disease [22, 25, 26].

Risk Factors for Alcoholic Hepatitis and Cirrhosis

Alcohol Consumption

Before 1960, protein malnutrition was widely believed to be the cause of cirrhosis in heavy drinkers. Although nutritional deficiencies are common in these individuals, there is now agreement that alcohol rather than generalized malnutrition is the most important factor causing serious liver injury in heavy drinkers [27]. Many studies have shown a relationship between per capita consumption of alcohol and the prevalence of cirrhosis across years and countries (Figs. 1.3 and 1.5) [8, 28, 29]. However, in population and

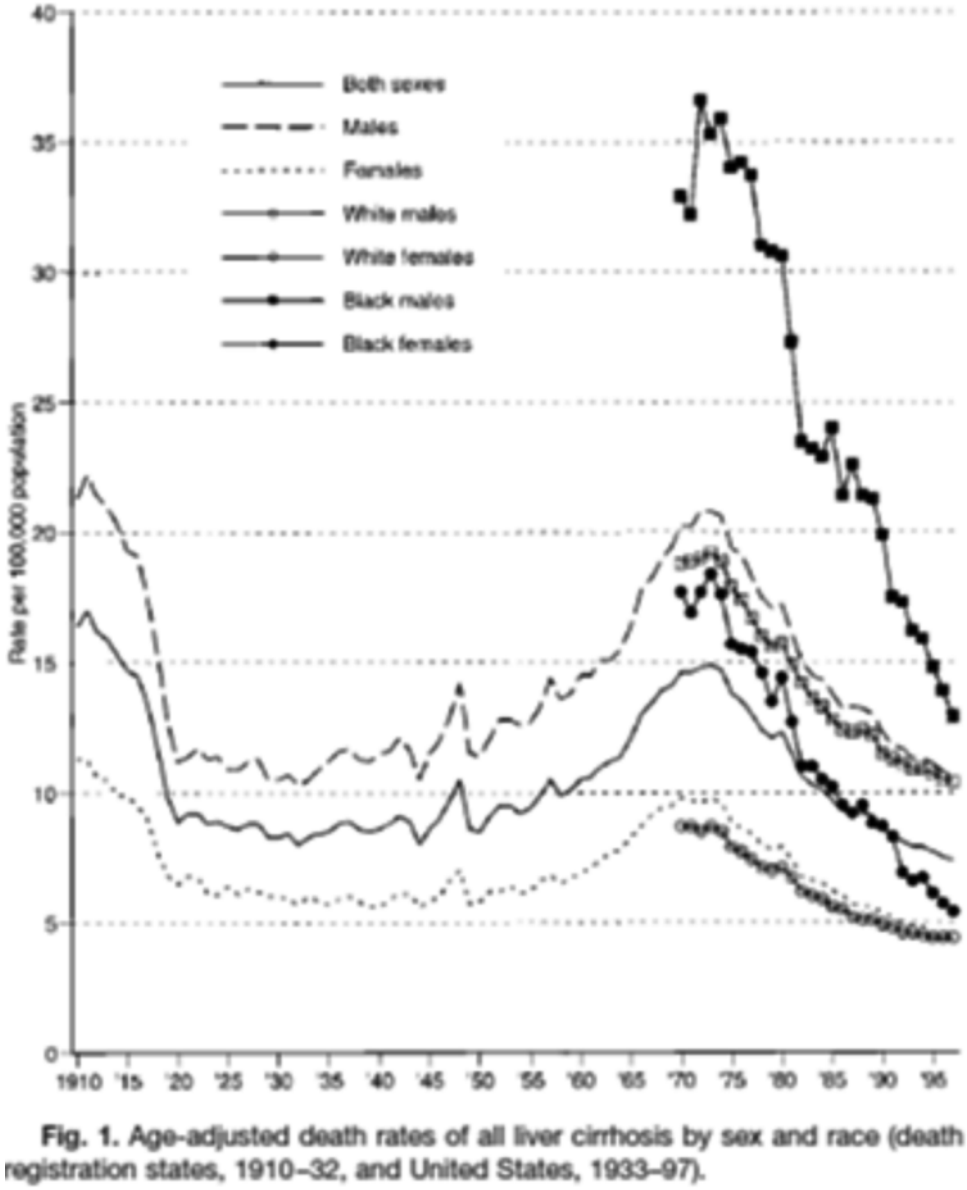

Fig. 1. Age-adjusted death rates of all liver cirrhosis by sex and race (death registration states, 1910–32, and United States, 1933–97).

Fig. 1.5 Age-adjusted cirrhosis mortality in the USA by sex and race, 1910–1995. From Stinson et al. Alcoholism Clinical and Experimental Res, 2001, p. 1181. Cirrhosis mortality rates in the USA decreased substantially between 1910 and 1920 presumably as a result of prohibition that ended in 1919. Although an increase was observed between 1950 and 1970, the rate returned in 1995 to levels observed during prohibition

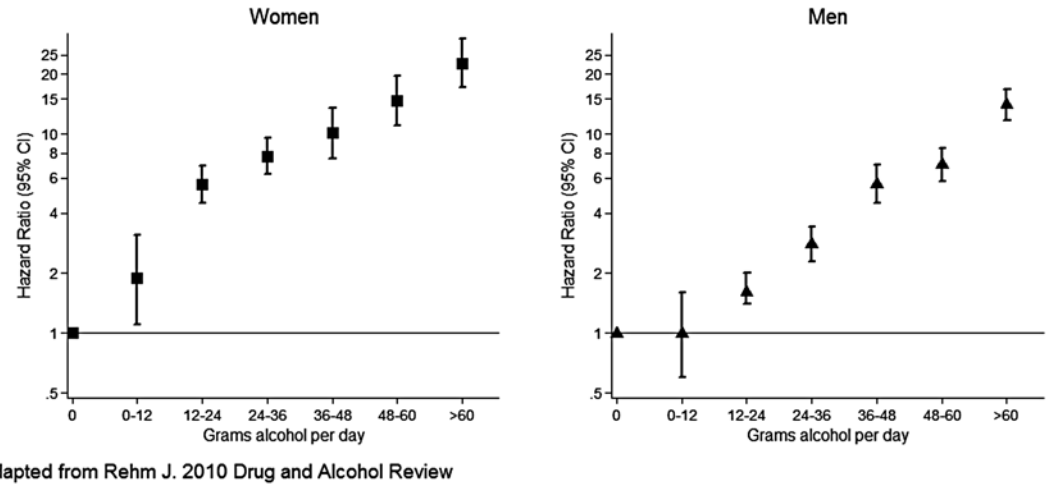

Fig. 1.6 Dose–response relationship between alcohol consumption and cirrhosis mortality by sex. Data from a meta-analysis illustrate the nearly linear relationship between dose and cirrhosis mortality rate. The cirrhosis mortality rate for women is increased compared to that for men and is particularly evident at lower levels of consumption, between 12 and 24 g daily

case-control studies, the absolute risk of serious liver injury in heavy drinkers is surprisingly low, ranging from 6 to 15 % [30–32]. Data from the Dionysos study estimated the prevalence of alcoholic cirrhosis to be 0.43 % in the population, accounting for approximately 38 % of all cases of cirrhosis in the study population [31]. The absolute risk of developing alcoholic cirrhosis was calculated to be 9.8 % in those consuming more than 60 g of ethanol daily, which is similar to studies from Copenhagen that reported an absolute risk of 6 % [30] in subjects drinking more than 35 drinks/week (~60 g daily). By contrast, a population study of 1270 Chinese drinkers estimated an absolute risk of 14.6 % in those drinking more than 40 g daily. Two prospective studies in Chinese showed an increased risk of cirrhosis with a threshold of 20 g daily drinking [32–34], whereas the threshold appears to be at least 30–60 g in Western studies [30, 35, 36]. Whether the difference between these two populations is related to ethnic or genetic differences in susceptibility or to differences in accurate reporting of alcohol consumption is unclear.

In almost all studies, the risk of more serious forms of ALD and cirrhosis mortality increases with higher daily consumption of alcohol [8, 35, 36] (Fig. 1.6). However, very heavy drinkers and those who are sick with acute alcoholic hepatitis are often underrepresented in prospective population studies making it difficult to determine the true increase in risk. Patients admitted to hospital with alcoholic hepatitis and cirrhosis reported mean daily alcohol intake of 170–220 g [15, 37]. Finding a dose–response relationship is consistent with a direct causal effect of alcohol, but the relatively low prevalence of advanced disease even in heavy drinkers indicates that additional factors such as genetics, patterns of consumption, diet, and other factors are important contributors to the overall risk.

Duration of Heavy Drinking

Most patients with serious alcoholic liver disease manifest signs and symptoms in the 4th to 5th decades of life, although occasionally, subjects in their 20s may present with florid alcoholic hepatitis. Determining the duration of heavy consumption is problematic since many heavy drinkers vary their intake over the course of the lifetime. The mean duration of heavy drinking averaged 20 years in patients with alcoholic liver

disease compared to 13 years in those with alcoholic pancreatitis [37]. A steady increase in the cumulative incidence of cirrhosis and non-cirrhotic liver disease was observed beginning around age 45–50 in the Dionysos study [31]. Changes in cirrhosis mortality rates were correlated with changes in per capita alcohol consumption during prohibition in the USA (Fig. 1.5) [8, 28, 38]. Cirrhosis mortality rates were also associated with changes in alcohol consumption in Europe during the 1980s–1990s but the temporal relationship was not sufficiently clear to define a specific lag period [39]. While it is clear that fatty liver can develop within a relatively few days of heavy consumption, more serious stages of ALD such as alcoholic hepatitis and cirrhosis develop over a much longer interval of time. Since serious alcoholic liver injury such as alcoholic hepatitis and cirrhosis occurs infrequently even in those who drink very heavily for many years, a number of other factors likely contribute to the overall risk.

Patterns of Consumption and Beverage Type

Whether alcoholic hepatitis and cirrhosis develop more often in binge drinkers or in those who are daily drinkers is unclear. Drinking outside mealtime was associated with a higher risk of cirrhosis and non-cirrhotic liver disease in the Dionysos study [31]. Danish drinkers who drank only wine had a lower risk of cirrhosis than beer and spirit drinkers which is of interest since in Denmark, wine is consumed primarily at mealtime [35]. These results must be interpreted cautiously since cirrhosis mortality rates are relatively high in France, a predominantly wine-drinking country (Fig. 1.2), making it likely that the pattern of consumption rather than beverage type is key. The duration of alcohol consumption (16–17 years) was similar in Spanish drinkers with alcoholic steatosis, hepatitis, or cirrhosis, but an irregular pattern of drinking (mealtime and between mealtimes) was far more likely in patients with alcoholic hepatitis with or without cirrhosis than in those with either cirrhosis alone or steatosis alone [40]. The authors also noted that patients with severe alcohol withdrawal syndrome (SAWS) on admission to the hospital were more likely to have irregular patterns of consumption than those without SAWS. Although some have speculated that the substantial rates of binge drinking both in North America and in Europe may eventually lead to an increase in the risk of developing more severe ALD, so far, the data are inconclusive. Clearly more screening and surveillance efforts are needed to detect any trend; in addition, preventive efforts to decrease the level of alcohol consumption in the whole population (including heavy drinkers) seem warranted.

Gender

Women are at greater risk for development of serious liver injury including alcoholic hepatitis and cirrhosis than men based on equivalent intake of alcohol. Both prospective, population-based and case-control studies have identified a threshold of 30–80 g of ethanol daily in men and 20–40 g in women above which the relative risk of serious alcoholic liver disease is significantly increased [31, 32, 37, 41]. A meta-analysis by Rehm and colleagues illustrates the lower threshold for increased risk of ALD in women compared to men (Fig. 1.6) [36]. Other studies noted similar mortality rates from alcoholic cirrhosis in women and men but the relative risk of hospitalization for alcoholic cirrhosis was higher in women than in men [8]. Anecdotally, the frequency of severe, acute alcoholic hepatitis seems to be higher in women than in men. Many potential mechanisms have been proposed to explain the increased risk in women. The lower threshold of drinking required to develop alcoholic hepatitis and cirrhosis in women may, in part, be related to differences in body weight and/or composition.

The increased risk may also result from differences in metabolism of ethanol in women compared to men. ADH activity is influenced by sex hormones [42]. Although gastric metabolism of ethanol may be lower in women than in men, it is unlikely that the difference in metabolism accounts for the increased risk of alcoholic liver disease in women.

Nutritional Factors: Protein, Vitamins, and Minerals

Although protein/calorie malnutrition was once believed to be the cause of liver disease in heavy drinkers, there are no compelling data to support this conclusion [27]. Surveys of patients with advanced liver disease showed adequate dietary protein intake above the recommended daily requirements in the vast majority. In one study, a high-fat diet was correlated with the risk of cirrhosis in heavy drinkers [41]. Other studies noted differences in the incidence of cirrhosis that were related to the source of animal protein most often consumed [43]; however, this observation remains unconfirmed.

Many specific nutrient deficiencies are common in patients with alcoholic cirrhosis including B vitamins and some minerals, particularly zinc [44]. Zinc deficiency increases gut mucosal permeability by disrupting tight junctions, allowing translocation of bacteria and other pathogen-associated molecular patterns [45]. Recent studies have noted differences in gut mucosal permeability in patients with serious alcoholic liver injury, particularly alcoholic hepatitis, suggesting a role for the "leaky gut" in the pathogenesis of alcoholic hepatitis. To our knowledge, no RCT has evaluated the role of zinc among people with ALD for the prevention of cirrhosis.

Vitamin A deficiency is often associated with zinc deficiency. Both are common in heavy drinkers particularly those with liver disease [46]. Unfortunately, large doses of vitamin A are hepatotoxic and the combination of ethanol and vitamin A supplementation is more toxic, making guidelines about replacement of vitamin A challenging in heavy drinkers [46]. Although high intake of vitamin A was associated with a higher risk of cirrhosis in an Italian case-control study, there was no additive effect of alcohol and vitamin A intake [47]. By contrast, lower intakes of riboflavin and vitamin B_{12} were associated with an increased risk of cirrhosis in the same population.

Interaction Between Alcohol Consumption and Obesity

Fatty liver, steatohepatitis, and cirrhosis can all occur in obese individuals who do not drink any alcoholic beverages, usually referred to as nonalcoholic fatty liver disease (NAFLD). Accumulating evidence has linked obesity to an increased risk of all the hepatic manifestations of ALD (namely, steatosis, hepatitis, cirrhosis), cirrhosis mortality, and decompensation of cirrhosis [16, 48–50]. However, studying the interaction between alcohol and obesity on liver disease is complex.

From an operational perspective, many studies linking obesity with cirrhosis (presumably due to NAFLD) have segregated the study population based on the level of alcohol consumption. The studies of NAFLD by definition exclude excessive drinking. Therefore, very few studies with wide ranges of obesity and alcohol consumption have examined any manifestation of liver disease. National surveys that include BMI, alcohol, and liver enzymes, liver steatosis by ultrasound, or liver-related mortality probably provide the best evidence for an association [49, 51, 52].

In the ALD literature, a large study of heavy drinkers, who underwent biopsies upon admission to hospital, identified obesity as one of the five variables independently associated with alcoholic cirrhosis [16]. All subjects were consuming at least 50 g of ethanol daily. Of the 1604 subjects included, 411 had cirrhosis with or without acute alcoholic hepatitis. In addition to obesity, the duration of heavy consumption and female gender were identified as independent risk factors for both acute alcoholic hepatitis and cirrhosis. Other studies have also identified obesity as an independent risk factor for cirrhosis in heavy drinkers [49, 51].

Although the risk of steatosis is increased in obese, heavy drinkers, it has been suggested that the risk of fibrosis may not be greater in obese subjects who drink small amounts of alcohol [20]. These findings, however, are in disagreement with another study [53]. Clearly, the relationship

between alcohol consumption and obesity in the risk of advanced liver disease and cirrhosis is complex, since light to moderate consumption of alcohol may improve insulin sensitivity and other metabolic parameters that reduce the development of NAFLD, whereas heavier consumption may be additive with NAFLD in the risk of progression to cirrhosis.

Alcohol and Iron Overload

Hepatic iron overload is seen frequently in patients with alcoholic cirrhosis [54–58].

Iron overload predicted mortality in patients with alcoholic cirrhosis but not cirrhosis from hepatitis C [59]. However, the relationship between iron overload and alcoholic cirrhosis is complex. Studies of a well-characterized population of patients with genetic hemochromatosis (C282Y homozygotes) showed that patients whose alcohol intake was >40–60 g daily had a ninefold higher than expected risk of cirrhosis [60]. Since there was not a control group of subjects without iron overload, comparing the risk in this population to the risk of cirrhosis in subjects consuming similar amounts of alcohol without genetic hemochromatosis is difficult. Similar findings were observed in another population of patients with genetic hemochromatosis [61]. Interestingly, high intake of dietary iron was associated with cirrhosis but was independent of alcohol intake [47].

In animal studies, feeding a high-iron diet with alcohol increases lipid peroxidation and fibrosis [58]. Non-transferrin-bound iron levels are significantly elevated in active drinkers but not in those who have advanced alcoholic liver disease who are abstinent [62], while serum transferrin levels are elevated in subjects with fatty liver due to heavy drinking but low in patients with alcoholic cirrhosis [63]. Serum ferritin levels are increased even in moderate drinkers but serum ferritin poorly predicts hepatic iron concentration even in heavy drinkers [58, 64]. The mechanism underlying so-called secondary iron overload in heavy drinkers is unclear.

While there is good evidence to show that any alcohol intake above 40 g daily in men leads to an increased risk of cirrhosis, there is relatively little compelling evidence that heterozygosity for C282Y or H63D increases the risk of alcoholic cirrhosis [60]. Although secondary iron overload is common in patients with alcoholic cirrhosis, the extent of hepatic iron overload is far less than is seen in genetic hemochromatosis and probably does not contribute significantly to the development of liver injury.

Alcohol and Hepatitis C

Both heavy alcohol consumption and hepatitis C are common causes of cirrhosis in Europe and North America. Although hepatitis C can lead to cirrhosis even in nondrinkers, heavy alcohol consumption is a significant risk factor in the progression of fibrosis and development of cirrhosis in patients with hepatitis C infection [65–71]. Studies have also shown a higher prevalence of advanced fibrosis or cirrhosis in patients with hepatitis C infection who drink more than 50–80 g of ethanol daily [72]. Both the lifetime consumption of alcohol and the amount of alcohol consumed following infection with HCV were associated with the increased risk of cirrhosis in HCV patients, whereas the mean daily intake at the time of the study was not related [67]. Although data from Italy did not show an increased risk of cirrhosis in those with lifetime daily drinking less than 50 g daily, the authors reported that higher daily intake increased the odds of cirrhosis in those with HCV and that the effects of heavy alcohol consumption and HCV infection are synergistic rather than additive (Table 1.1) [71]. One meta-analysis provided an estimated pooled relative risk for cirrhosis of 2.33 in those drinking more than 30–80 g of ethanol daily [73]. The increased risk for cirrhosis in patients with hepatitis C is more evident in men than in women, a finding that is somewhat surprising since women are at higher risk of cirrhosis due to heavy drinking compared to men, as described before. One explanation for the

Table 1.1 Odds ratios for cirrhosis according to lifetime daily alcohol intake and anti-HCV status

Lifetime daily alcohol intake	Anti-HCV negative	Anti-HCV positive	S index
None	1.0	9.2 (2.0–43.2)	
25 or 50 g/day	0.9 (0.5–1.6)	9.5 (4.3–21.2)*	1.1 (<0–2.4)
75 or 100 g/day	4.5 (2.1–9.3)*	26.1 (8.7–78.2)*	2.2 (0.6–3.7)
125 or 150 g/day	13.0 (5.7–29.4)*	133.4 (37.6–473)*	6.5 (4.4–8.7)*
≥175 g/day	15.0 (7.1–31.7)*	147.2 (42.1–514)*	6.6 (4.5–8.7)*

*$p<0.05$

S index is defined as $(OR_{AB}\text{-}1)/(OR_{AC}+OR_{AD}\text{-}2)$ where OR_{AB}=OR in anti-HCV positive drinkers of a specific dose of alcohol; OR_{AC}=OR in anti-HCV positive teetotalers and OR_{AD}=OR in anti-HCV negative drinkers of a specific dose of alcohol; OR is crude observed odds ratio

Data are adapted from Corrao and Arico [71]

difference is that fewer heavy drinking women were represented in the studies of patients infected with HCV.

Some studies have shown that the risk of progression of chronic hepatitis C is increased when there is fat seen on liver biopsy. Obesity and diabetes are risk factors for progression of chronic hepatitis C to cirrhosis. Genotype 3 hepatitis C is associated with hepatic steatosis as well as an increase in the risk of developing type 2 diabetes independently of BMI. Therefore, it is highly plausible that fatty liver and inflammation due to hepatitis C may act in a synergistic manner in development of fibrosis and cirrhosis in these patients.

While most guidelines for management of chronic hepatitis C recommend abstinence, whether the risk of cirrhosis in moderate drinkers with chronic hepatitis C infection is increased is a subject of controversy [74]. One study of 78 patients with paired liver biopsies showed increased progression of fibrosis in patients drinking less than 40 g daily [75]. However, several larger studies have shown that the risk of cirrhosis in patients with HCV infection is not increased in light to moderate drinkers (<30 g daily) [47, 76, 77]. Data from these large studies casts doubt on whether progression is more likely to occur in light to moderate drinkers, particularly those who drink less than once weekly.

Alcohol and Hepatitis B Infection

Even though heavy alcohol consumption increases the progression of chronic hepatitis C to cirrhosis, there is very little evidence that alcohol consumption has a similar effect on the progression of hepatitis B. Unlike chronic hepatitis C, the pathogenesis of inflammation in chronic hepatitis B is closely related to the host immune response to the infection. This difference may be important in explaining the difference between the effects of alcohol consumption on hepatitis B and C. Most studies have been carried out in Asia where the prevalence of chronic hepatitis B is high and the consumption of alcoholic beverages is relatively low. These cultural differences may account for the lack of evidence of an interaction. Even though consumption of alcoholic beverages is increasing in Asia, widespread vaccination and subsequent reduction in the incidence of hepatitis B infection may make demonstrating a potential association more challenging in the future.

Genetic Factors

Differences in susceptibility to complex diseases among individuals are often due to inherited traits. Early studies showed an increase in the rate of concordance for alcoholic cirrhosis in monozygotic versus dizygotic twins [78]. Tremendous advances in understanding how single-nucleotide polymorphisms influence risk of disease or response to exogenous factors now help to explain why some individuals are more susceptible while others are inherently resistant. Although genetic risk factors are unlikely to explain all of the individual differences in the incidence of chronic diseases such as alcoholic liver disease, they are undoubtedly important risk factors [79]. Ultimately, the interaction between genes that increase risk for ALD and environ-

mental exposure (consumption) to ethanol is likely responsible. Both genes that increase the probability of heavy consumption (alcohol use disorders) and genes that increase the probability of liver injury in those who drink heavily are important contributors to the overall risk for an individual.

Alcohol-Metabolizing Enzymes: ADH, ALDH, and Cytochrome P450 IIE1

Alcohol dehydrogenase (ADH), the primary family of enzymes responsible for metabolism of ethanol, was one of the first enzymes purified. Five classes of ADH as well as genetic polymorphisms that determine the rate of metabolism and elimination of ethanol have been identified [80]. Furthermore, genetic polymorphisms in the rate of metabolism of ethanol and acetaldehyde are associated with differences in consumption of alcoholic beverages. For example, some East Asians have an aversive reaction to drinking alcoholic beverages characterized by flushing and nausea. These individuals have a high frequency of ADH3*1, an isoenzyme that rapidly metabolizes ethanol to acetaldehyde and a high frequency of a catalytically inactive form of aldehyde dehydrogenase, ALDH2*2 [81, 82]. Homozygosity for ALDH2*2 results in moderately severe intolerance for alcohol with flushing above a minimal level of consumption. Asians with this phenotype have a low incidence of alcohol use disorders, perhaps because of the aversive effects of acetaldehyde accumulation after drinking alcohol. In one European study, ADH2*2 was associated with a lower risk of alcoholism [83], whereas another study, also from Europe, failed to show any association of ADH polymorphisms with either alcoholism or ALD [84]. A subsequent meta-analysis showed an increased risk of alcoholism associated with ADH2*1 and ADH3*2 [85]. The effects were most pronounced in East Asians but were also noted in Caucasians. These alleles code for isoenzymes with a lower catalytic rate than ADH2*2 and ADH3*1, suggesting that a high rate of elimination of alcohol may protect against developing alcohol use disorders, particularly in East Asians with the ALDH2*2 allele.

Because total ethanol consumption is a known risk factor for liver disease, genetically determined differences in consumption of alcoholic beverages can influence the risk of developing alcoholic liver disease. The frequencies of ADH2*2 and ADH3*1 were both lower in Chinese patients with alcoholic cirrhosis [86], whereas the frequency of ADH2*1 was higher [87–89]. Meta-analysis comparing heavy drinkers with and without ALD did not show any association with polymorphisms of ADH2 or ADH3 [85]. Although these polymorphisms may influence drinking behavior, they do not appear to contribute to the risk of ALD in heavy drinkers except indirectly through total consumption.

The catalytically inactive ALDH2*2 allele was found in lower frequency in patients with alcoholic cirrhosis [82]. Not surprisingly, the frequency of the ALDH2*1 isoenzyme with normal catalytic rate was higher in Chinese patients with alcoholic cirrhosis [89]. However, a meta-analysis showed ALDH2*1 to be associated with alcoholism but not with alcoholic cirrhosis [85].

Polymorphisms exist in the Pst I/Rsa I region of cytochrome p4502E1. The frequency of the c2 allele of Pst I/Rsa I was reported to be higher in Japanese men with alcoholic cirrhosis [89, 90]. However, no difference in the gene frequency was observed in Caucasians with ALD [91]. or Chinese patients with alcoholic cirrhosis [87]. Furthermore, no differences in cyp 2E1 were observed in either Japanese or Caucasian patients with alcohol abuse [91, 92]. A meta-analysis failed to show a specific relationship between the c2 allele and alcoholic liver disease [93]. However, an odds ratio of 3.12 was observed for homozygosity for c2c2 versus c1c1 comparing alcoholics with liver disease to alcoholics without liver disease. A second polymorphism of cyp 2E1 (Dra I) also exists. However, no association with alcoholism or ALD has been observed [93]. Since cyp 2E1 is an enzyme that is induced by ethanol consumption, the inherent activity of the various polymorphisms may not be as important as the response to induction, which is more challenging to measure and may be related to many other factors.

In conclusion, polymorphisms in ADH and ALDH influence levels of consumption of alco-

holic beverages and the rates of alcoholism particularly in East Asians, but there is no particular association of these polymorphisms with the risk of developing ALD in heavy drinkers. Polymorphisms in cytochrome P450 are unlikely to contribute either to risk of alcoholism or ALD.

Genes Involved in Oxidative Stress

Oxidative stress has been hypothesized to play a role in the pathogenesis of alcoholic liver injury. Based on this hypothesis, a number of candidate gene studies were conducted in the last 20 years. Polymorphisms in several genes involved in oxidative stress other than cytochrome P450 2E1 have been studied in ALD. One study from France suggested that homozygosity for alanine rather than valine in the mitochondrial targeting domain of manganese superoxide dismutase (MnSOD) increased the risk of microvesicular steatosis, alcoholic hepatitis, and cirrhosis but not macrovesicular steatosis [94]. Unfortunately the findings were not confirmed in subsequent studies from the UK or Portugal [95, 96].

Genes Related to the Innate Immune System

A single-nucleotide polymorphism, −159 C>T, in the promoter region of the endotoxin-binding receptor CD14 was associated with an increase in the risk of ALD in three small studies [97–99] but not in other studies [95, 100]. A meta-analysis of 8 studies that included 1083 patients with ALD, 548 alcoholics without liver disease, and 1140 control subjects showed an increase in the risk of cirrhosis in patients with TC versus CC and for TT/TC versus CC, compared to alcoholics without liver disease [101]. No increase in risk was observed for other forms of ALD such as steatosis.

A functional polymorphism −159 C>A in the promoter region of the anti-inflammatory cytokine IL-10 was also associated with an increase in risk of ALD in one study from the UK but not confirmed in others [102–104]. A meta-analysis did not show an increase in the risk after the pooling of the data from the three studies [104]. However, the small sample sizes may not be adequate to detect a difference in patients with ALD from alcoholics without ALD.

A number of studies have examined two polymorphisms in the promoter region for TNF-alpha. Grove and colleagues originally reported an association between the G>A substitution at position 238 and alcoholic steatohepatitis [105]. They did not find an association with the polymorphism at the 308 position. These findings were confirmed in a meta-analysis of 11 studies with an odds ratio of 1.47 for association of the −238 TNF promoter with alcoholic cirrhosis but not with the broader definition of all forms of ALD [106].

In addition to TNF, levels of IL-1 are elevated in patients with alcoholic hepatitis and cirrhosis [107]. One study showed an association of the −511 IL1 beta allele 2 with development of alcoholic cirrhosis in Japanese men [108], but this observation remains unconfirmed by other studies.

Genes Related to Lipid Metabolism, Particularly PNPLA3

In 2008, a genome-wide association study (GWAS) of participants in the Dallas Heart Study discovered that an allele, rs738409G, in the patatin-like phospholipase known as PNPLA3 (adiponutrin) was strongly associated with increased hepatic fat levels [109]. Although there was a clear association with increased hepatic triglycerides, there was no association with BMI, insulin resistance, or other parameters frequently associated with NAFLD. Of note, the frequency of the allele was higher in Hispanics and lower in African Americans, consistent with the known risk of fatty liver in these populations. Additional studies indicated that the same allele was associated with elevated ALT in other populations suggesting that it may be related to the progression of liver disease as well as accumulation of fat [110]. A second variant that is also associated with NAFLD was discovered through exome-wide association study of subjects in the Dallas Heart Study [111]. The TM6SF c499A>G allele encodes a protein whose function is unclear. Its association with NAFLD is greater in Caucasians of European descent than in Hispanics or African Americans.

PNPLA3 is postulated to be involved in triglyceride hydrolysis. Expression of the mutant

form (I148M), but not the wild type, in cultured hepatocytes or in the livers of mice resulted in accumulation of triglyceride within the cells [112, 113]. The I148M substitution inhibits the catalytic activity of the enzyme and limits hydrolysis of triglyceride. Additional work is required to fully understand the target of PNPLA3 and how the mutant interferes with hydrolysis of triglyceride. PNPLA3 is secreted from hepatocytes and circulates in plasma but there is no known function or target for the circulating enzyme [114].

Following the initial discovery of the strong association of rs738409G with fatty liver, the potential association of this SNP with ALD was evaluated. Tian and colleagues found a strong, highly significant association with both ALD (OR 1.45) and alcoholic cirrhosis (OR 2.25) in a Mestizo population of mixed Hispanic/European descent [115]. Soon after this publication, three additional studies from Europe and UK confirmed similar strong associations with alcoholic cirrhosis and non-cirrhotic ALD [116, 117]. A meta-analysis of 11 published studies that included subjects from India as well as Europe and Mexico confirmed a strong association of the G allele either as a dominant or recessive model (Table 1.2) [118]. To date, this association is the strongest and most consistent predictor of genetic risk for alcoholic liver disease and cirrhosis.

Table 1.2 Odds ratios for alcoholic cirrhosis compared to controls in GG versus CC genotype

Publication	Odds ratio	95 % Confidence interval
Tian et al. (2009)	4.44	2.77–7.10
Seth et al. (2010)	5.27	1.55–17.96
Falleti et al. (2011)	6.95	4.03–11.99
Nguyen-Khac et al. (2011)	3.79	0.87–16.57
Nischalke et al. (2011)	8.38	3.73–18.84
Stickel et al. (2011)	3.60	1.95–6.64
Trepo et al. (2011)	2.18	1.26–3.78
Rosendahl et al. (2012)	5.74	3.40–9.66
Dutta et al. (2013)	4.31	1.66–11.15
Way et al. (2013)	2.71	1.53–4.79
Total	4.30	1.25–5.69

Data are adapted from Chamorro et al. [118]

Since the mutant form of PNPLA3 (I148M) is associated with more advanced forms of alcoholic liver disease, there may be other functions of PNPLA3 than hydrolysis of triglyceride that permits or facilitates progression of the disease after triglyceride accumulation in response to ethanol.

Concluding Remarks

Development of liver damage and cirrhosis related to heavy drinking is complex. The medical paradigm has largely focused on sick or high-risk individuals. This thinking has shaped most of the research questions, planning of health expenditures, and provision of care. For example, we have continuously focused on answering the following question: Why did this individual develop liver disease? Heavy consumption of alcoholic beverages can accelerate liver damage in some diseases such as chronic hepatitis C and genetic hemochromatosis. There is also substantial evidence to suggest a genetic risk for liver damage in heavy drinkers as well as a genetic risk for drinking heavily as outlined above. Risk factors for ALD such as obesity are also genetically determined, leading to a complex model for understanding the risk of developing ALD. GWAS and exome association studies of alcoholic liver damage are needed to confirm results of some candidate gene studies. Fortunately, prior studies are valuable in eliminating certain factors such as generalized malnutrition, chronic hepatitis B infection, and probably the type of alcoholic beverage consumed.

Importantly, factors that explain the incidence of disease within a population and the risk factors for disease within an individual are not necessarily the same. These differences are now becoming more apparent in an era in which genetic risk is both identifiable in individuals and potentially quantifiable within a population. Understanding the individual genetic risk factors will help to identify those populations that may be at higher or lower risk for disease based on alcohol consumption. This complex interaction between genes and the environment is a subject of intense

interest and further investigation in the study of liver injury due to alcohol.

Future epidemiological studies will need to account for the effects of modifiable risk factors such as the amount, duration, and pattern of consumption of alcoholic beverages in genetically defined populations, such as those with different genotypes for ALD risk factors. For example, based on our current understanding, it is highly likely that an obese woman with the GG genotype for rs738409 will develop cirrhosis at a lower threshold of alcohol consumption than a lean man with the CC genotype. Careful studies of the patterns and levels of consumption of alcoholic beverages in genetically well-characterized populations are needed to confirm the tantalizing results of the last 50 years of investigation into the pathogenesis of cirrhosis related to alcohol consumption. Once these results are obtained, sound advice about the risk associated with consumption of alcoholic beverages can be provided to individuals. We then need to ask ourselves, why does this population have so much disease [119]? Public health practitioners will then be able to provide an understanding of how to effectively prevent or reduce the burden of disease within a defined population based on these and other characteristics of the population.

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Global Epidemiology and Risk Factors for Nonalcoholic Fatty Liver Disease

2

Abhijit Chowdhury and Zobair M. Younossi

Epidemiology of NAFLD in North America and Europe

Prevalence The epidemiology of NAFLD in the USA and Canada (North America) is similar, and the rates from these two countries can be interchangeable [1–7]. The data from the USA estimates that 27–34 % of the general population have NAFLD while 75–92 % of the morbidly obese individuals have NAFLD [8]. Additionally, prevalence of NAFLD patients with type 2 diabetes is high with a prevalence rate estimated to be between 60 and 70 % [9]. As the prevalence of obesity and metabolic conditions increased over the past two decades, the prevalence of NAFLD continues to rise [6, 10].

In the USA, there are ethnic differences for the prevalence of NAFLD. In fact, the prevalence of NAFLD among European-Americans is 33 %, while it is 45 % in Hispanic Americans and 24 % in African Americans [3–7]. These data seem consistent from different studies from the USA reporting the highest prevalence of NAFLD in Hispanic Americans and lowest prevalence in African Americans [3–7].

Similar to North America, the prevalence of NAFLD in Europe is also very high. In fact, one-fourth of the general European population may have NAFLD with the prevalence rates reported as low as 8 % from Romania and up to 45 % from Greece [7, 11–13]. Although not entirely clear, the wide range of prevalence rates is most likely due to the NAFLD definitions and the diagnostic modalities used [14, 15].

Again, similar to the US patients with diabetes, prevalence of NAFLD in the European patients with diabetes is also high ranging between 42.6 and 69.5 % [16]. Furthermore, the prevalence of NAFLD in patients who meet the criteria for metabolic syndrome rate has been estimated to be about 79 % [17] (Table 2.1).

Incidence There is no precise data on the incidence rates for NAFLD in North America or Europe. This is partly due to the fact that NAFLD is usually a silent disease discovered incidentally. Nevertheless, given that the prevalence of obesity in adult Americans has almost doubled since the early 1960s (1962—48 % vs. 2010—75 %), the incidence of NAFLD in the USA has almost certainly increased [32, 33].

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Table 2.1 Prevalence of NAFLD in the USA and Europe

Author, year	Country	Population	Sample size (*n*)	Prevalence of NAFLD (%)	Mode of diagnosis	Ref. No.
Price, 2014	USA	HIV+ and HIV− men in the Multicenter AIDS Cohort Study.	*N* = 719 *n* = 254 HIV-men *n* = 465 HIV + men	Overall-15 % HIV− = 19 % HIV+ = 13 %	Fatty liver was defined as a liver-to-spleen attenuation ratio < 1 on non-contrast computed tomography (CT).	[18]
World Gastroenterology Organization Global Guidelines, 2012	USA	A representative sample of the US population	NA	General population 27–34 % Morbid obesity 75–92 % European-Americans 33 % Hispanic Americans 45 % African Americans 24 %	A variety of sources not reported	[19]
Ruhl, 2003	USA	NHANES III (1988–1994)	5724	5.9 %	ALT > 43 U/L	[20]
Clark, 2003	USA	NHANES III (1988–1994)	15,676	10.3 %	ALT > 40 U/L and AST > 37 U/L for men and AST > 31 U/L for women	[21]
Browning, 2004	USA	Dallas Heart Study	734 (non Hispanic white only)	33 %	Hepatic triglyceride content >5.5 %	[5]
Ioannou, 2006	USA	NHANES (1999–2002)	6823	9 %	ALT > 43 U/L or AST > 40 U/L	[22]
Lazo, 2013	USA	NHANES III (1988–1994)	12,454	19 %	Ultrasound	[23]
Schneider, 2014	USA	NHANES III (1988–1994)	4037	25.1 %	Ultrasound	[6]
Giday, 2006	USA	Local clinic data	320	19 %	Liver biopsies	[24]
Williams, 2011	USA	Cohort of middle age patients at large hospital	328	46 %	Ultrasound	[25]
Bedogni, 2005	Italy	Dionysos Project	3345	20 %	Ultrasound	[26]
Radu, 2008	Romania	Cohort of hospitalized patients	3005	20 %	Ultrasound	[27]
World Gastroenterology Organization Global Guidelines, 2012	Europe	A representative sample of the European population	NA	20–30 %	A variety of sources not reported	[1, 19]
Gastaldelli et al., 2009	14 EU countries	Healthy subjects between 30 and 60 years	1400	33 %	Fatty liver index	[28]
Haring, 2009	Germany	Subjects aged 20–79	4160	30.4 %	Ultrasound	[29]
Zois, 2010	Greece	Subjects aged 3–98 years old	498	31 %	Autopsy	[30]
Caballeria, 2010	Spain	Individuals aged 15–85 years old	773	33.4 %	Ultrasound	[31]

Parallel to the increase of obesity in North America, the rate of obesity in European countries has also increased. In 2008, the rate of obesity throughout Europe was estimated to be 15.5 % with another 34.6 % of general population recorded as being overweight [19]. It is important to note that there may be some geographic difference in rates of obesity in Europe. In fact, the prevalence of obesity was found to be highest in Hungary (28.5 %), the UK (26.1 %), and Ireland (23.0 %) followed by Malta (22.7 %), Luxembourg (22.7 %), and the Czech Republic (21 %) with lowest rates being reported from Romania (8.5 %), Switzerland (8.7 %), Norway (10 %), and Italy (10.2 %). Given that these prevalence rates for obesity throughout Europe are increasing, the incidence of NAFLD in Europe is expected to rise. Despite this paucity of data on the incidence of NAFLD in North America and Europe, the rates from outside these two areas have been estimated to be around 10 % per year [34].

Risk Factors

Age, Gender, and Ethnicity

Clinical characteristic of patients with NAFLD are similar in North America and Europe [31, 33–43]. The average age of NAFLD patients in North America and Europe is 40–50 years old. Contrary to the initial description of NASH, the majority (60–70 %) of patients with NAFLD in the USA are male. Similar gender distribution is reported from Europe except for specific countries such as Lithuania where most of the NAFLD patients are reported to be female [10, 35].

As previously mentioned, most (90 %) patients with NAFLD are found to be overweight or obese. Additionally, age may be associated not only with the development of NAFLD but also its progressive form, NASH [36]. In one particular study, patients who developed NASH were younger, were of Hispanic origin, and had components of metabolic syndrome [37]. These findings were supported in another study where NAFLD/NASH patients were more likely to be male ($P<0.0001$); have lower hip-to-waist ratios ($P=0.03$); were less likely to be African American ($P=0.06$); and had higher levels of alanine aminotransferase (ALT; $P<0.0001$), aspartate aminotransferase (AST; $P<0.0001$), and serum triglycerides ($P=0.0154$), but lower levels of high-density lipoprotein cholesterol ($P<0.0001$). In this study, patients with NAFLD who had moderate to severe fibrosis were older ($P=0.0245$), were more likely to be male ($P=0.0189$), were Caucasian ($P=0.0382$), have diabetes mellitus ($P=0.0238$), have hypertension ($P=0.0375$), and have a lower hip-to-waist ratio ($P=0.0077$). Furthermore, they had higher serum AST ($P<0.0001$) and ALT ($P<0.0001$) levels. After multivariate analysis for predicting moderate to severe fibrosis in NAFLD patients, the significant independent variables were male sex, Caucasian ethnicity, diabetes mellitus, and increased AST and ALT levels (model P value <0.0001). Based on these findings the investigators developed a predictive model to help clinicians identify patients at high risk for developing or of having advanced fibrosis. This model had a positive predictive value 31.4 % (23.9–39.8 %) and a negative predictive value of 91.0 % (86.7–94.3 %). It is important to note that presence of diabetes alone increased the odds of having advanced fibrosis 25.41 %. This risk incrementally increased as other metabolic syndrome components were added. For example, for patients who had diabetes and hypertension, their risk for moderate to advanced fibrosis was 26.32 %, and if patients also had central obesity (lower hip-to-waist ratio), their odds increased to 26.67 % [38]. It is important to note that in addition to the high prevalence of NAFLD and NAFLD-related fibrosis in diabetics, NAFLD patients with diabetes are also at risk for increased liver-related mortality [38–44].

Although NAFLD may be more common in men, female patients with polycystic ovary syndrome (PCOS) have an increased risk for NAFLD. Researchers have found that the prevalence of both polycystic ovary syndrome

and nonalcoholic fatty liver disease rises proportionally to the degree of insulin resistance and the mass of adipose tissue present [45]. The mechanism of action that may cause this association or in fact may actually increase the progression of NAFLD in women with PCOS may be reflected by significantly elevated levels of caspase-cleaved CK18 (M30) suggesting a more proapoptotic environment. This, in addition to the hyperandrogenic state present in PCOS, may cause a suppression of the LDLR (plays a major role in the clearance of apoB- and apoE-containing lipoproteins) receptor sites both in adipocytes and in the liver creating a prolongation of the half-life of VLDL and LDL thereby causing steatogenic effects [45, 46].

In summary, NAFLD in North America and Europe is associated with obesity, diabetes, and other component of metabolic syndrome, including PCOS in female patients. Additionally, being male, being younger (<50 years old), and being of Hispanic descent in the USA increase the risk of having NAFLD. Although lean NAFLD can be seen in these parts of the world, they represent a much smaller cohort with a different clinical profile [37].

BMI, Obesity, and NAFLD

As noted previously, risk factors for the development of NAFLD reported from North America and Europe include components of the metabolic syndrome (obesity, dyslipidemia, hypertension, and diabetes/insulin resistance) [31, 41, 47–51]. In this context, visceral obesity is the most important predictor of outcome in NAFLD. In fact, in one study, visceral adipose tissue (VAT) as measured by computed tomography was shown to be strongly associated with NAFLD [(HR) 2.04:1.23–3.38] [52].

The data confirming the association of NAFLD with obesity come not only from tertiary care center but also from population-based studies [2–7, 9, 11–18, 20–67]. In a study from the USA which included 3056 NHANES participants, NAFLD patients were found to be older with a higher BMI, larger waist circumference, and higher sum of skinfolds and had insulin resistance (HOMA > 3.0) or type 2 diabetes [66].

In Europe, similar risk factors for NAFLD (type 2 diabetes, obesity, hypertension, and dyslipidemia) are reported from Europe. In fact, obesity remains the most prevalent risk factor for NAFLD in Europe with 65–90 % in patients with NAFLD being obese or overweight. Data recently reported from 165,000 adults who were included in the report from the European Commission on Fatty Liver Inhibition of Progress (FLIP) suggested higher BMI, waist circumference, weight gain during adult life, and physical inactivity all will increase the risk of each stage of clinically recognized NAFLD (REF). Furthermore, both arterial hypertension and dyslipidemia were highly prevalent in patients with NAFLD, especially in women [67].

Given the interactive association of NAFLD with obesity, both obesity and NAFLD should be considered as similar complex disorders which are related to the environment and genetic predisposition. The environmental factors influencing obesity and NAFLD are related to dietary intake (both number of calories and composition of these calories), activity, degree of stress, cultural issues, and other potential contributors [68]. The genetic predisposition of NAFLD is very also interesting and will be discussed in detail in subsequent chapters.

As noted previously, it is important to mention that NAFLD in the USA may also be present in nonobese patients. In fact, this type of NAFLD may be more common in Asian countries. In the USA, the prevalence of NAFLD among lean subjects was estimated to be only 3.7 %, while this rate was 17.7 % in the obese and overweight individuals [37]. Furthermore, the clinical profile of lean patients with NAFLD is also different where the patients tended to be younger, female, and having a decreased likelihood of having insulin resistance and hypercholesterolemia [37].

Insulin Resistance, Metabolic Syndrome, Diabetes, and Cardiovascular Disease in NAFLD

As noted previously, patients having a history of diabetes 2 or insulin resistance, dyslipidemia, and hypertension are at increased risk for the

development of NAFLD. In one study, the risk of having NAFLD was highest for persons with diabetes (OR, 4.16; 95 % CI, 3.24–5.33), followed by presence of metabolic syndrome (OR, 3.97; 95 % CI, 3.26–4.83). Among other components of metabolic syndrome, central obesity was associated with highest odds for presence of NAFLD (OR, 3.41; 95 % CI, 2.77–4.20) as well as severity of NAFLD (OR, 5.58; 95 % CI, 3.86–8.06). The more component of metabolic syndrome, the higher the risk of NAFLD. In fact, the odds of having NAFLD when three components were present was 9.49 (95 % CI, 5.67–15.90), and when five components were present, the odds was 24.05 (95 % CI, 12.73–45.45) [49].

Given the common risk factors between NAFLD and cardiovascular diseases (CVD), there are increasing reports for higher rates of CVD and CV mortality in NAFLD [1, 49, 69]. A recent study investigating the relationship of NAFLD and cardiovascular disease found that the odds for having a carotid intima–media thickness [cIMT] > 0.8 mm and/or presence of plaques in obese patients with NAFLD was 5.96 (95 % CI, 1.60–22.25; $p = 0.008$) in men and 8.26 (95 % CI, 4.02–16.99; $p < 0.001$) in women [69]. In addition to high rate of CVD, there is also an increased rate of CV mortality. Although liver disease has been reported as the third leading cause of death among persons with NAFLD, cardiovascular disease and malignancy are the two top causes of death in NAFLD patients [34]. In one particular long-term follow-up study (mean follow up time of 18.5 years), approximately 60 % of the patients with NAFLD had died with the most frequent cause of death cited as coronary artery disease (30 %), followed by nonliver malignancy (18 %) and then liver-related mortality including hepatocellular carcinoma (15 %) [38, 44].

The strong association of DM and metabolic syndrome with NAFLD has also been reported from Europe. In a prospective study of 230 patients from nine centers, metabolic syndrome was found in the majority of patients (53 %) with 54 % of the patients being male with a mean age of 49.4 ± 13.9 years and a mean BMI of 30.6 ± 4.6 kg/m^2. In 16 % of the patients, undiagnosed diabetes was discovered. For the patients (51 %) who had a liver biopsy, fibrotic staging was significantly more severe in patients with metabolic syndrome (2.43 ± 1.25 vs. 1.73 ± 1.18, $p < 0.001$). A subgroup of patients with GGT > 5 × ULN were significantly older (55.9 vs. 47.64 years, $p = 0.02$), were more frequently diabetic (53 % vs. 23 %, $p = 0.01$), and had more advanced fibrosis (3.42 vs. 1.08, $p = 0.0080$ [41].

Another interesting study from Europe was initiated in Italy. The Italian Society for the Study of Atherosclerosis (SISA) in 2005 started a research project aimed to study NAFLD. Using ultrasound (US) in nondiabetic subjects, the researchers set out to determine the prevalence of NAFLD, its associated risk factors and prevalence of hypertransaminasemia, and its possible determinants. NAFLD prevalence was 0.78. Their initial results showed that men with hepatic steatosis (as compared to men without steatosis) were younger ($P < 0.05$) and had higher triglycerides ($P < 0.03$), higher homeostasis model assessment insulin resistance (HOMA-R) ($P < 0.003$), and increased visceral fat thickness ($P < 0.0001$). Furthermore, women with steatosis showed higher triglycerides ($P < 0.05$), HOMA-R ($P < 0.04$), VFT ($P < 0.0001$), and younger age ($P < 0.05$). Multivariate analyses found that visceral fat thickness ($P < 0.0001$), HOMA-R ($P < 0.02$), and triglyceride to HDL ratio ($P < 0.05$) were associated with the severity of NAFLD. Age ($P < 0.05$), log ratio of triglycerides ($P < 0.005$), and visceral fat thickness ($P < 0.01$) were also associated with higher ALT. The prevalence of steatosis was reported to be the highest reported in patients with metabolic syndrome. These investigators concluded that due to the exclusion of severely obese and diabetic patients, their findings highlight the prominent role that alterations of lipid metabolism play in the pathogenesis of NAFLD [17].

Finally, in a recent study from Finland, the prevalence of NAFLD in young Finnish was 29 % in overweight/obese and 5 % in normal weight individuals. The independent correlates were waist circumference, ALT, BMI, male gender, triglycerides, systolic blood pressure, and insulin resistance [70].

Diet and Physical Activity

Although there seems to be a genetic component to the predisposition to NAFLD, diet and activity play a major role. It seems the Western diet high in calories and refined sugar and fructose may play a role in the development of obesity and associated NAFLD. Nevertheless, systematic assessment of diet and activity at the population level in the USA is scarce [71–82].

Other dietary components may also play a role. In one large population-based study using 4 cycles of NHANES data from 2001 to 2008, investigators studied dietary intake questionnaires which listed 62 nutritional components. Univariate analysis found that 38 % of the nutritional components were significantly different between patients with NAFLD and those without NAFLD where patients with NAFLD. After multivariate analysis adjusting for demographic confounders (age, gender, ethnicity), Hispanic race, being male, being obese (BMI > 30), and drinking less caffeine were associated with NAFLD. Although the issue is controversial and the mechanism is not entirely clear, drinking caffeine in the form of coffee actually seems to have a protective effect on the development of NAFLD. This is possibly due to the suppressive effect that coffee has on hyperglycemia by improving insulin sensitivity through the reduction of inflammatory cytokine expression [71, 72].

It is important to note that diet can have an impact both by being responsible for the development of central obesity as well as a direct effect on the inflammatory environment of patents with NAFLD. In fact, one of the most exciting areas of research in NAFLD is the contribution of visceral adipose tissue (VAT) to the development of NAFLD. In fact, the white adipose tissue of VAT is thought to be an endocrine organ that produces adipokines and cytokines responsible for the development of an inflammatory milieu contributing to pathogenesis of NASH, CVD, and other complications of visceral obesity [73]. In addition to VAT being responsible for the inflammatory milieu of NAFLD, there is also some contribution of diet itself on these proinflammatory fat cytokine/chemokine expression within the liver. Animal studies have suggested that diets high in saturated fat, fructose, and cholesterol as well as a specific combination of carbohydrates and fats (starch/oleate) set off a cascade of molecular metabolic derangements including insulin resistance and activation of the miRNA resulting in a more severe form of NAFLD or NASH [74–76]. In addition to adipocytokines, oxidative stress also plays a major role in the development of NAFLD. Recent findings suggest that the extent of hepatocyte ballooning reflect the severity of oxidative DNA damage and accumulation of DNA methylation in NAFLD [77, 78]. In fact, the impact of diet on the oxidative stress cycle has been suggested [78, 79]. Diet can influence microRNA which is involved in the pathogenesis of NAFLD. In fact, animals exposed to a high fat diet showed a dysregulation of miRNA-451 leading to development of NAFLD and fibrosis [77, 78, 80, 81]. All of these mechanisms may be related to the "additional" effect of diet on the development and progressive nature of NAFLD.

In regard to physical activity, patients with NAFLD seem to have low activity levels compared to controls. In fact, patients with both NAFLD and diabetes experienced the lowest level of physical activity [82].

In summary, NAFLD is associated with components of metabolic syndrome. Although genetic predisposition probably plays a role, it only explains a small portion of the increased risk. In this context, the influences of environmental factors are significantly more important. Metabolic syndrome is quite frequent in the general population, although its prevalence varies considerably according to the criteria used for its definition. Additionally, metabolic syndrome is associated with NAFLD, with the WHO definition being the best to determine its presence, probably because of the inclusion of insulin resistance as a main component. Unification of criteria for metabolic syndrome is needed to adequately compare its prevalence and relationship with NAFLD in different population groups [31].

Epidemiology of NAFLD in Asia

Nonalcoholic fatty liver disease (NAFLD) is an emerging health-care priority in Asia [83–85]. This has a potential impact not only for the emerging liver disease burden in this region but also as a broader public health issue in view of the association of NAFLD with the other metabolic syndrome (MS)-linked noncommunicable diseases (NCD)—obesity, diabetes, and atherosclerotic cardiovascular disease. These countries are in a state of health-care transition with the emergence of a new set of public health priorities that have MS as the unifying factor. NAFLD and the other NCDs are key determinants in this changing disease burden scenario that have implications on global health [86].

Socioeconomic affluence and changes in lifestyle influence NAFLD prevalence in a population. The Asian countries, with their large population, are passing through a period of rapid economic growth and shift of focus in labor policy from a dominant physical to one that depend on knowledge capital and foster physical inactivity. An increasing GDP in these nations is paralleled by a rising body mass index (BMI)—the most widely used surrogate of obesity—in an almost linear relationship to GDP growth [87, 88]. An expanded body fat mass and insulin resistance (IR) are the hallmarks of each of these different MS-linked conditions which frequently coexist, although one may antedate the other [89]. In the light of all these, it is imperative that epidemiology of NAFLD be studied in the context of a broader systemic disease perspective, rather than as a liver disease only.

Overall, NAFLD prevalence in this most populous region of the world is high and is increasing over time. There are several particular features of NAFLD in Asia that need specific mention.

Firstly, Asians have also been shown to have an increased propensity to the adverse clinical outcomes in MS-linked noncommunicable diseases (NCD) including coronary atherosclerosis, diabetes, and hepatic steatosis than other ethnic groups in general [90, 91].

Secondly, Asian people often develop NAFLD, metabolic syndrome, and diabetes in the context of anthropometric parameters, usually measured as BMI, that are considered subthreshold as health risks in Western populations. As a consequence, a different set of BMI and waist circumference cutoffs have been proposed as correlative with MS-linked health risks, including NAFLD, among the Asians [92–94].

Thirdly, the contribution of NAFLD in overall liver disease burden in this region has to be seen in the background of an already existing high prevalence of chronic viral hepatitis and a spiraling increase in per capita alcohol consumption in the general population of the region. Synergism in liver disease progression among various etiologies does exist, and this add further complexities in assessment of impact of NAFLD in the emerging liver disease burden in Asia and the Middle East [95–97].

Fourthly, Asian population groups are ethnically diverse. In addition, there are national as well as regional imbalances in socioeconomic development and cultural changes in this region—each of which influences the heterogeneity of the available data of NAFLD epidemiology that are available from Asia. The study design—characteristics of the population sample and the methodology for diagnosis of NAFLD—also differs significantly across studies. Despite these, the available data provide an assessment of NAFLD epidemiology in Asia and the Middle East with fair precision and depict the changes happening over time.

Prevalence

Most of the available epidemiological studies in NAFLD from Asia are ultrasound based and hence detect prevalence of hepatic steatosis alone initially, correlating it with anthropometric, biochemical, and demographic features of the population (Table 2.2). A more detailed workup, including measurement of liver stiffness—(continued attenuation parameter based quantification of liver fat, CT scan and liver biopsy) has been carried out in a few of these studies for better precision and characterization of liver disease status. A more robust radiological approach was based

Table 2.2 Prevalence of NAFLD in Asian countries

Author, year	Country	Population	Sample size (*n*)	Proportion of nonobese among NAFLD subjects	Prevalence of NAFL[a]	Prevalence of MS/ diabetes among NAFLD persons	Mode of diagnosis	Ref. no.
Fan et al., 2005	Shanghai, China	General population (urban)	3175	NR	611 (19.24 %)	NR	US	[98]
Wong et al., 2012	Hong Kong, China	General population (urban)	922	NR	264 (28.6 %)	125 (47.34 %)[b]	MRS	[99]
Chen et al., 2006	Taiwan	General population (rural)	3245	61 (16.39 %)[c]	372 (11.5 %)	346 (93 %)[b]	US	[100]
Park et al., 2006	Korea	Hospital OPD	6648	419 (33.79 %)[c]	1240 (16.1 %)	234 (18.87 %)[d]	US	[101]
Omagari et al., 2002	Nagasaki, Japan	Hospital OPD	3432	141 (44 %)[c]	319 (9.3 %)	47 (14.7 %)[d]	US	[102]
Jimba et al., 2005	Japan	Hospital health checkup	1950	NR	566 (29 %)	(10.95 %)[d]	US	[103]
Amarapurkar et al., 2007	Mumbai, India	Selected population (railway colonies)	1168	48 %[c]	16.6 %	22 %[d]	US	[104]
Das et al., 2010	West Bengal, India	General population (rural)	1911	90 (54 %)[e]	167 (8.7 %)	43 (26 %)[d]	US and CT	[105]
Vendhan et al., 2014	Chennai, India	General population (urban)	541	48 (27.74 %)[f]	173 (32 %)	NR	US	[106]
Dassanayake et al., 2009	Sri Lanka	General population (urban)	2985	305 (31 %)[c]	974 (32.6 %)	(49.17 %)[d]	US	[107]
Niaz et al., 2011	Karachi, Pakistan	Tertiary care hospital	952	NR	129 (13.6 %)	NR	ALT and US	[108]

US ultrasound, *MRS* magnetic resonance spectroscopy, *CT* computed tomography, *NR* not reported

[a]Unadjusted prevalence

[b]Prevalence of metabolic syndrome

[c]Nonobese defined as BMI <25 kg/m^2

[d]Prevalence of abnormal Fasting blood glucose

[e]Nonobese defined as BMI <23 kg/m^2 and waist circumference <80 cm (female)/<90 cm (male)

[f]Nonobese defined as BMI <23 kg/m^2

on MR spectroscopic quantitative estimation of hepatic triglycerides in two studies from Hong Kong. The major variables for study quality were the stringency methods adopted in selection of the study population to remove bias and the method of estimating NAFLD since the majority of the studies included select populations from those attending clinics or from the workplace [8, 84, 109]. There have also been well-planned general population-based studies from India, China, Taiwan, Hong Kong, Korea, and Japan that have used standard sampling strategies with stratifications [98–106, 108, 110–117].

While the strength of the studies differ, NAFLD prevalence in Asia is high (15–20 %) and is increasing over time [84, 118, 119]. There is wide variation in NAFLD prevalence. In general, the prevalence is higher in select, clinic-based populations and lower in the general population studies and higher in urban than rural population studies (Table 2.2). Far Eastern countries (China, Korea, Hong Kong, and Japan) report a higher prevalence in the population than South Asian countries (India, Sri Lanka).

In Asia, the largest number of epidemiological studies is available from China where the prevalence of NAFLD is 20 % (6–38 %). Similar prevalence estimates are available from Japan (15 %), Korea (16–22 %), Hong Kong (Proton MR spectroscopy—27 %), and Taiwan. Data from India is more diverse as are the quality of the reported studies (8–30 %). The prevalence is at least 10 % of the population. Data from Sri Lanka, Malaysia, and Indonesia also indicate that ultrasound prevalence of NAFLD is around 15–20 % of the general population [107, 120–122].

Temporal trends of BMI over the past three decades indicate a progressive global upslope that is marked in the Far East, but is comparatively flat in South Asia [87]. NAFLD has also shown a similar increase in prevalence in the last three decades at least in China and Japan, from where data is available [94, 123]. Of greater importance in Asia is the fact that at least 15–20 % of NAFLD subjects (as high as 54 % in one study) may have a BMI that is within the normal limits for clinical risk, although subtle alterations in markers of increased fat mass in the body may be present. Waist circumference and waist–hip ratio has been shown to be a more useful marker of obesity and metabolic risk including NAFLD correlations among these "metabolically obese normal weight (MONW)," variably called "nonobese"/"lean" NAFLD subjects [37, 124–139].

Incidence

Despite the fairly large body of data on the prevalence of NAFLD from the Asian population, information on incidence (new-onset NAFLD in people previously free from it over a given time period) is relatively scarce (Table 2.3). However, there are a few well-designed population cohort follow-up studies that report an annual incidence of 3–5 % and most importantly point out the dynamic nature of the process of hepatic steatosis, documenting resolution or regression in another 5 % of subjects. Weight gain, even within the normal range, with increments in BMI and other markers of adiposity along with presence and worsening of MS markers had consistently been shown to be associated with NAFLD incidence in Asian population. Incident fatty liver is often associated with new development of hypertension, ALT increments occur in people who develop incident NAFLD, and there is evidence that weight loss can reverse this dynamic state of hepatic steatosis.

While prevalence and incidence of NAFLD in the adult population increase with aging, a greater concern is the rapid increase of childhood obesity and NAFLD in Asian countries. The prevalence of obesity among children and adolescents is increasing in most Asian countries, and this might occur at a more rapid pace than that in the West. NAFLD–NASH in children is important not only as a liver disease burden but also as an important predictive determinant for development of vascular—endothelial risk in the Asian population, later in life. Reports of NAFLD prevalence among children and adolescents in Asia (Japan, Korea) and the Middle East (Iran, and Egypt) vary between 2.8 % and 15 % in cross-sectional studies. NAFLD in children increases with age and BMI, is more prevalent among boys

Table 2.3 Incidence of NAFLD in Asian countries

Author, year	Country	Population	Sample size (*n*)	Follow-up period	Incidence of NAFL	Mode of diagnosis	Risk factors for new NAFLD	Ref. no.
Wong et al., 2015	Hong Kong, China	General population (urban)	565	47 (34–60) months	78 (13.8 %); 13.5 % at 3–5 yr	MRS	Increase in WC and TG	[131]
Hamaguchi et al., 2005	Japan	Hospital health checkup	3147	414 ± 128 days	308 (10 %)	US	Presence of MS at baseline	[127]
Xu et al., 2013	China	Nonobese subjects[a]	5562	5 years	494 (8.8 %)	US	Age, gender, BMI, WC, TG, HDL, serum uric acid, Hb, and platelet count	[129]
Sung et al., 2014	South Korea	Hospital health checkup	11448	5 years (retrospective)	1418 (12.38 %)	US	Associated with new-onset hypertension	[130]
Donghee Kim et al., 2014	Korea	General population	1375	5 years	288 (20.9 %)	US	Increase in visceral adipose tissue	[132]
Pankaj Singh et al., 2014	West Bengal, India	Nonobese subjects in general population (rural)[b]	83	5 years	26 (31 %); 62.65 per 1000 person-year	US	Higher degree of adiposity at baseline and increase over time	[133]

US ultrasound, *MRS* magnetic resonance spectroscopy, WC waist circumference, TG triglyceride, BMI body mass index, Hb hemoglobin

[a]Nonobese defined as BMI <25 Kg/m^2

[b]Nonobese defined as BMI <23 Kg/m^2 and waist circumference <80 cm (female)/<90 cm (male)

than girls, has a similar distribution of central adiposity like their adult counterparts, and can lead to significant liver disease. Overnutrition with high-calorie, low-nutrient diet, and physical inactivity are factors associated with the prevalence of pediatric and adolescent NAFLD, while the consequences may be felt in terms of an increasing liver disease burden as well as diabetes and cardio-metabolic risk in the future.

As mentioned previously, most individuals with NAFLD are obese. In fact, obesity, measured by standard criteria for BMI and waist circumference along with its ethnicity-specific modifications, is undoubtedly the most significant association of NAFLD. However, it has emerged that a varying proportion of NAFLD subjects (15–21 %, up to 75 % in one study from India), from Asia, do not have obesity (BMI <25), despite having similar metabolic abnormalities (MS and IR) seen classically in obesity-associated NAFLD. A differential distribution of body fat with expansion of visceral adipose tissue compartment, recent increase in body weight, dietary factors including switch from a traditional carbohydrate-dominant to a cholesterol- and saturated fat-dominant diet, genetic factors predicting unique predispositions, and possibly a different gut microbiome may all be contributing to this subphenotype of NAFLD in Asia [124, 140, 141]. Asians, particularly South Asians, have been shown to have a lower mean insulin sensitivity and higher values of HOMA-IR and hepatic triglycerides for a similar value of BMI as compared with Caucasians, black, and Hispanic people. In addition, adipocytokine profile has been shown to be different in them with higher values of IL-6 indicating a relatively heightened degree of inflammatory activation. In addition, South Asians have larger adipocytes along with higher leptin and lower adiponectin values compared to Caucasians [91]. A similar and related entity described in general MS literature is called metabolically obese normal weight (MONW), and nonobese/lean NAFLD may be the hepatic counterpart of "sick fat cell syndrome" seen in obesity and insulin-resistant states [124]. Despite the anthropometric differences, the very tight link of nonobese NAFLD with insulin resistance and metabolic syndrome suggests that it is not a biologically different entity. Follow-up of a prospective cohort of 155 stringently selected nonobese (BMI <23 Kg/m^2 and waist circumference <80 cm in female/<90 cm in male) subjects, free from NAFLD at baseline, reported a cumulative 5-year incidence of NAFLD to be 31 % [28]. Higher degree of adiposity at baseline and higher increments in anthropometric indices over time were correlated with the development of fatty liver in those without a baseline [133]. In another multiethnic international study that compared "lean" with overweight and obese NAFLD subjects, there was significant phenotypic variability in each category across countries. It appears that ethnicity and regional environmental modifiers might be playing a role in the disease expressions where IR is the key biological determinant [142].

Risk Factors

The risk factors for NAFLD development in Asia, in general, are similar to that of the Western populations. There are subtle differences particularly with regard to the role of central obesity and possible genetic influences in determining the ethnicity-specific differences in body fat partitioning and predisposition to outcomes of NAFLD and its MS-linked cardio-metabolic consequences [143–155].

Age and Gender

In all populations, prevalence of NAFLD along with severity NASH-related liver disease, rate of progression of liver fibrosis, and development of hepatocellular carcinoma (HCC) increases as age advances. MS-linked comorbidities and non-hepatic complications of NAFLD also show increments with age. It is likely that this increased prevalence and severity of IR-linked conditions including NAFLD are due to the age-related decline in insulin sensitivity that has been observed in all populations [93, 109]. In Asia, NAFLD prevalence starts rising after 30 years of

age, with a near linear increase as age group advances. However, these are mostly based on data from adult studies. An important facet of changing NAFLD epidemiology is an increasing prevalence of NAFLD in childhood and adolescence [84, 137]. In the light of this, age-specific prevalence of NAFLD is likely to change in near future with an earlier peak age and a less steep upslope in NAFLD prevalence, changing NAFLD epidemiological features.

Males outnumber females in most studies of NAFLD from Asia, except among postmenopausal women where this difference disappears. Males have also been shown to have a higher degree of NASH, more severe liver fibrosis, and higher mortality in NAFLD, as compared to females. In general, three quarters of NAFLD subjects in some multiethnic studies from the West that included Asians were males, while this proportion is much less (44 %) among Caucasians. Epidemiological studies from Asia also reveal the prevalence of NAFLD to be higher in males than females.

BMI, Obesity, and NAFLD [88, 92–94]

Prevalence of NAFLD, as the hepatic manifestation of metabolic syndrome, bears a linear relationship with the prevalence of obesity in the population. BMI, as the most widely used measure of body fatness, correlates well with NAFLD and other MS-linked conditions—diabetes, hypertension, and atherosclerotic vascular disease. In NAFLD, an increasing BMI has been shown to be associated not only with increased prevalence and incidence but also with more severe liver disease—NASH and liver fibrosis. Weight loss with a reduction of BMI has also been shown to improve liver histology and outcome of NAFLD. It has emerged, however, that the strength of BMI as a marker of total adiposity has ethnicity-specific connotations and the current cutoffs for obesity and overweight may not be useful indicators of clinical risk of obesity-related disorders, including NAFLD, in Asian populations. Asians, as compared to other ethnic groups, have been shown to have a higher prevalence of MS for similar grades of BMI, a disproportionate propensity to develop vascular disease and diabetes for any amount of weight gain at a given BMI, and a regional body fat partitioning that fosters more fat in visceral areas (visceral adipose tissue—VAT) as compared to total body adiposity, and as a result BMI may be a suboptimal measure for adiposity in Asians. The implication for NAFLD is that a person who is not overweight or centrally obese by the Western criteria, such as the Third National Health and Nutrition Examination Survey (NHANES III), might still have excessive amounts of adipose tissue (defined as >15 % of total tissue), particularly visceral adiposity. VAT has been shown to be biologically different as compared to subcutaneous adipose tissue in that it is more inflammatory and is associated with more aggressive MS-linked disease outcomes, including NAFLD and its vascular associations. This has prompted a revision of the BMI cutoffs for health risks for Asian populations with a lower value in each category (normal 18.5–22.9 and obesity > 27.5 kg/m^2, instead of 30 in the Western populations) [92]. In addition, a waist circumference criteria (male >90 cm and female >80 cm) is widely used as useful anthropometric measures of central obesity in Asians. It has been shown that weight gain and increase in anthropometric indices of fatness, even while within the normal body weight and BMI range, might be producing fatty liver among Asians. Another aspect of this intriguing and complex relationship between body composition and NAFLD among Asians is the fact that ectopic fat depots might also contribute to the overall process of adiposity–MS–NAFLD–vascular disease relationship in Asians, and this needs to be looked into in larger focused studies [138, 143, 144].

Insulin Resistance, Metabolic Syndrome, Diabetes, and Cardiovascular Disease in NAFLD [84, 89, 94, 97, 113, 114, 127]

These conditions form a spectrum and insulin resistance (IR) holds key to the metabolic

syndrome (MS)-associated clinical outcomes. Varying degree of IR is present in two-thirds of NAFLD patients in Asia and in more than 95 % of NASH subjects. The frequency and grade of IR increase with the severity of liver disease in NAFLD. IR is one of the factors associated with liver fibrosis progression in NASH, and histological improvements of NASH are associated with reciprocal changes in IR. Diabetes is the classical clinical expression of MS, and as such NAFLD is present in at least 50 % of diabetes patients from Asia. Although this is similar to that observed in other populations, the frequency of diabetes at NAFLD diagnosis is lower in Asians (10–15 %) as compared to others (25–40 %). However, family history of diabetes is often present in NAFLD in Asia and the risk of development of diabetes is increased four- to fivefold within 4–6 years of NAFLD diagnosis. There is a linear relationship between the prevalence of components of MS and the risk of NAFLD in the population of Japan, China, Hong Kong, Korea, and India. Apart from diabetes, there seems to be a clearly demonstrable relationship between incident NAFLD and new development of hypertension in Asian population. Much of the increased cardiovascular risk in Asia occurs in the context of this shared relationship among MS clinical counterparts. NAFLD has been shown to be a good predictor of endothelial dysfunction and atherosclerotic vascular disease individually in different Asian populations, even in nondiabetic, non-hypertensive subjects. This expands the value of NAFLD detection as a general health risk in the population, beyond that posed through more traditional MS-linked associations in Asia.

Diet and Physical Activity [144–148]

NAFLD is an outcome of overnutrition and a result from an imbalance between intake of foods dense in calories and expenditure of energy through physical activity. The Asian countries are passing through a phase of differential but steadily progressive economic growth. This has an impact on the dietary habits and the social as well as cultural practices with increasing trend to adopt a Westernized lifestyle. Increasing urbanization and lesser dependence on jobs that necessitate physical labor along with increasing fat, protein, and calorie intake and availability of energy dense nutritionally imbalanced "fast" foods that have skewed nutritional values together with lesser intake of vegetables are factors that promote the nutritional–metabolic imbalance that culminates in MS and NAFLD. Among the nutritional components, cholesterol intake has been distinctly shown to be linked to NAFLD, while the role of fructose, complex carbohydrates, and micronutrients in Asian diet as contributors to NAFLD needs more focused studies. Further evidence for a role of diet and physical activity in NASH in Asian population has come from intervention studies. A Korean study undertook and evaluated the effects of exercise and diet modification on histological severity of steatosis in 120 living liver donors, only 59 % of whom were overweight or obese. Lifestyle modification for 12 weeks achieved weight reduction in 77 % of patients and steatosis improvement in 86 %; reduction of serum total cholesterol >10 % and weight >10 % were strongly related to major improvements in steatosis.

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Pathogenesis of Alcoholic Liver Disease

3

Gavin E. Arteel and David W. Crabb

Introduction

Alcoholic liver disease (ALD) remains one of the most prevalent disorders of the liver and, with the appearance of extremely efficacious hepatitis C antivirals, will likely resume its place among the lead causes of death or transplantation from liver disease. Like most other manifestations of heavy alcohol use, ALD develops only after years of drinking, and serious disease occurs only in a minority of patients. This variability in susceptibility to ALD remains unexplained and suggests an interaction between host factors and the environment, in addition to the consumption of large amounts of alcohol.

The usual course of ALD is thought to run from asymptomatic fatty liver to alcoholic hepatitis (although not always clinically apparent) or to cirrhosis without clinical hepatitis. Current experimental animal research suggests that some degree of hepatic inflammation is likely always present in patients developing cirrhosis. That alcohol, and not malnutrition per se, can cause all of these stages was proven by the work of Charles Lieber, who showed that with several years of alcohol feeding in the setting of a nutritious diet, baboons developed fatty liver, alcoholic hepatitis, as well as cirrhosis [1]. It is likely that most if not all heavy-drinking individuals develop alcoholic fatty liver. Alcoholic hepatitis is the most florid manifestation of ALD, usually heralded by the development of jaundice, often with hepatic encephalopathy and other complications of chronic liver disease. While presenting somewhat acutely, it is the result of prolonged heavy drinking and is often accompanied by fibrosis or cirrhosis when the patient comes to medical attention. The prevalence of alcoholic hepatitis appears to have increased over the past decade [2]; it differs from the other forms of ALD in the presence of inflammation. The inflammatory infiltrate is more prominently composed of neutrophils, but many other sets of leukocytes, including T cells and NK cells, are involved in the pathogenesis, and the activation of macrophages is felt to be central to the process. Macrophage activation in turn results from a sensitizing effect of alcohol on the macrophages and hepatocytes, coupled with increased gut permeability, leading to increased concentrations of microbial products such as lipopolysaccharide (LPS) and other substances which interact with

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the pathogen-associated molecular pattern (PAMP) receptors of the toll-like receptor family, which result in an innate immune response. Ultimately, these interactions lead to the activation of the inflammasome, infiltration with other inflammatory cells, death of hepatocytes, inhibition of regeneration, and stimulation of fibrosis mediated by the hepatic stellate cells (HSCs). This chapter will review metabolic effects of alcohol on the liver that result in the pronounced disturbances in lipid and protein metabolism, the generation of cellular stress, and the roles of the innate immune system. The understanding of these effects is needed for new approaches to therapy, which are sorely needed for alcoholic hepatitis in particular, which has short-term mortality approaching 40 % in severe cases.

Metabolism of Ethanol and Its Role in Oxidative Stress

Alcohol is an unusual liver toxin both because it serves as food (a source of energy representing up to half of the daily energy requirement for heavy drinkers) and it is taken in quantities far exceeding the usual doses of other hepatotoxins. The liver is the major alcohol-metabolizing organ in the body, and this, plus the anatomic location of the liver astride the portal vein, dictates that the liver bears the brunt of injury from excessive intake. The pathways which metabolize alcohol reflect an evolutionary adaptation to dealing with alcohols present in food (the result of fermentation and generated by the microflora of the gut), much like the adaptation to numerous xenobiotics that are metabolized by cytochrome P450 enzymes in the liver.

Alcohol Oxidation Pathways

Alcohol is metabolized by NAD+-dependent oxidoreductases (alcohol dehydrogenases, ADH), by catalases, and by microsomal cytochrome P450 enzymes (CYP2E1) to acetaldehyde and subsequently oxidized to acetate largely by the mitochondrial aldehyde dehydrogenase (ALDH2). Polymorphisms of the ADH genes have been studied as possible risk factors for ALD. The *ADH2*2* allele is associated with reduced risk of alcohol abuse, but a higher risk for alcoholic cirrhosis than those with *ADH2*1* [3]. The oxidation of alcohol by ADH is unregulated, which means this pathway will dominate other energy-generating pathways when alcohol is taken in large amounts. This generates large amounts of NADH, which in turn inhibits fatty acid oxidation, and by way of "reductive stress" can increase the generation of ROS by the mitochondria (see below). It is interesting that many papers over the past decade have shown that interventions that target other signaling pathways discussed below have ameliorated steatosis without, apparently, blocking the bulk of alcohol metabolism by ADHs. Therefore, the role of the elevated NADH/NAD+ ratio per se as the cause of fatty liver is open to question.

Heavy alcohol use induces the activity of CYP2E1, initially named the microsomal ethanol oxidizing system (MEOS), by several folds. This enzyme, embedded in the endoplasmic reticulum, oxidizes ethanol in an NADPH-dependent fashion. Induction of this enzyme results from both stabilization of the protein against degradation and increased transcription of the gene. Polymorphisms in the CYP2E1 gene have not been convincingly linked to risk of alcohol abuse or ALD. CYP2E1-mediated oxidation of alcohol, while quantitatively less important for the clearance of alcohol from the bloodstream, directly generates ROS in the microsomal compartment and becomes a greater source of ROS with prolonged heavy drinking (see below).

Acetaldehyde Oxidation

While ALDH2 has a low K_m for acetaldehyde, there are detectable levels of acetaldehyde in the liver during alcohol intoxication. Acetaldehyde is implicated in many of the effects of alcohol on signaling pathways described below, as well as generates protein adducts that may impair protein function or create neoantigens, which could play a role in inducing an adaptive immune response against hepatocytes in alcoholic hepatitis. ALDH2 is polymorphic, with a dominant negative allele

(*ALDH2*2*) present in about 40 % of East Asians. This polymorphism strongly affects risk of alcohol abuse [4]; those individuals with one or two copies of the *ALDH2*2* allele have markedly reduced liver ALDH2 activity and develop an Antabuse-like reaction when they drink. While this substantially lowers their risk of developing alcohol abuse, it does not appear to increase the risk of ALD [3]; however, these individuals have much higher rates of esophageal cancer attributed to increased acetaldehyde exposure during moderate drinking [5].

The Coevolution of Oxidative Stress and Alcohol Toxicity Research

Research dating back decades [6] suggested that ALD was caused by altered nutrition. As mentioned above, this hypothesis was overturned by landmark work showing that alcohol causes direct hepatotoxicity independent of nutritional status [7], and Di Luzio and colleagues [8] proposed that lipid peroxidation contributes to ethanol-induced liver damage. These concepts paralleled other work in the oxidative stress field. In the late 1960s, with the discovery of the function of SOD as a catalytic reducer of $O_2^{\bullet-}$ to H_2O_2 [9], the concept that oxidants are produced by the cell under normal conditions gained hold. Discoveries of other antioxidant enzyme systems (e.g., peroxidases and catalases) and prooxidant enzymes (e.g., NOX enzymes, NOS isoforms, and myeloperoxidases) illustrated that prooxidants are often intentionally produced by the cell. Therefore, the concept that oxidative stress contributes to ALD coevolved with the current understanding of oxidative stress.

Mechanisms by Which Alcohol Causes Oxidative Stress

Any changes in the cell that favor prooxidant formation or disfavor antioxidant defenses can cause oxidative stress. Potential mechanisms by which ethanol increases prooxidant production are via electron leakage from normal biologic processes and increased activity of prooxidant enzymes. Ethanol also causes biochemical changes that favor generation and propagation of potent prooxidant species. Lastly, ethanol consumption can directly or indirectly impair defenses against prooxidants (discussed in section "Alcohol Interference with Antioxidant Defenses"). These effects are discussed in detail here, as they are relevant to all the subsequent sections.

Electron Leakage as a Source of Oxidative Stress

Enzyme-catalyzed transfer of electrons is critical for normal cellular function. Even with tightly coupled reactions, electrons can leak to other electron acceptors, such as oxygen. For example, the reduction of O_2 to H_2O by the mitochondria is not complete, and 1–2 % of O_2 consumption by mitochondria is due to the formation of $O_2^{\bullet-}$ under basal conditions [10]. Alcohol exposure increases the yield of $O_2^{\bullet-}$ from this cellular component in the liver (e.g., [11]). Elevated prooxidant production by mitochondria not only increases the net yield of prooxidants in the cell but can also directly damage mitochondrial proteins and DNA, which can exacerbate mitochondrial aging and stimulate mitochondrial-mediated apoptotic pathways (see [12] for review). Moreover, alcohol depletes mitochondrial GSH levels, which increases the response of hepatocytes to apoptotic stimuli [13]. Therefore, it is likely that prooxidant production from mitochondria is key for the development of oxidative stress caused by alcohol.

Molecular oxygen (O_2) is the recipient of electrons from ethanol during CYP2E1-mediated oxidation, and hydrogen peroxide (H_2O_2) is reduced to water by catalase. CYP2E1 is relatively loosely coupled with cytochrome P450 reductase; it can therefore leak electrons to oxygen to form $O_2^{\bullet-}$ or catalyze lipid peroxidation [14], which increases with CYP2E1 induction by alcohol. This isozyme has also been shown to be induced in macrophages; macrophages overexpressing CYP2E1 have a more robust response to stimulation in culture [15], which may contribute to the "priming" effect of alcohol on these cells (see section "Priming of the Innate Immune System in ALD").

Prooxidant Metabolites of Ethanol

A major prooxidant produced from alcohol metabolism acetaldehyde, which is present in micromolar concentrations in the liver after alcohol consumption. Acetaldehyde is highly electrophilic and can form adducts with reactive residues on proteins or small molecules (e.g., cysteines). Furthermore, acetaldehyde enhances glutathione (GSH) utilization and turnover and depletes the reduced pool of GSH [16], which is critical to maintain catalytic antioxidant defenses. These chemical modifications can also alter and/or interfere with normal biologic processes and be directly toxic to the cell [17]. Modified biologic molecules may also stimulate the host immune response and cause an autoimmune-like disease. Antibodies against acetaldehyde-modified proteins have been reported in both humans and animal models of alcohol exposure [18]. For example, a hybrid adduct of malondialdehyde and acetaldehyde (MAA) unique to alcohol exposure has been shown to induce an immune response both in human alcoholics and in animal models of alcohol exposure [19].

Other products of alcohol metabolism may also induce oxidative stress in organs. In addition to oxidative metabolism, ethanol can also undergo metabolism via a non-oxidative pathway, in which the end products are fatty acid ethyl esters (FAEE [20]). These molecules are thought to accumulate in the mitochondria of cells and to potentially uncouple oxidative phosphorylation and thereby increase ROS production; although a minor metabolism pathway in the liver, they are suspected to contribute to tissue damage [21].

Production of Prooxidants from Prooxidant Enzymes

Inappropriate activation of inflammatory cells plays a key role in the initiation of alcoholic liver injury (see section "Priming of the Innate Immune System in ALD"). Inflammatory cells are "professional" producers of ROS and reactive nitrogen species (RNS) such as $NO^{\bullet}$. Whereas the production of these species is critical for host defense, if inappropriately stimulated, they can also cause damage to normal tissue. Two major sources of prooxidants in these cells are NAD(P)H oxidase (NOX2) and the inducible form of nitric oxide synthase (NOS2). In experimental ALD, genetic ablation of NOS2 almost completely protected the liver against alcoholic liver damage [22]. Interestingly, whereas RNS production from NOS2 may be damaging in alcohol-induced organ injury, the activity of endothelial NOS (NOS3) is often protective; the different isoforms of NOS may therefore serve different functions in the disease state [23]. Other sources of ROS include lipoxygenases and myeloperoxidases. These cells permit the generation of additional oxidative compounds. H_2O_2 can react with Cl^- via myeloperoxidase in neutrophils to make $HOCl^-$ [24]. The enzyme-independent reaction of $O_2^{\bullet-}$ with $NO^{\bullet}$ form $ONOO^-$, a strong oxidizing and nitrating species [25].

Although electron leakage from normal enzymatic processes is arguably the dominant source of parent ROS in noninflammatory liver cells, these cells also possess prooxidant-producing enzymes, which may contribute to organ damage caused by alcohol. Oxidative stress plays a role in the transformation of HSCs into myofibroblasts, the critical matrix-producing cell in the fibrotic liver [26]. The contribution of oxidative stress to HSCs' transformation is not solely in response to extrinsic ROS, but also from intracellular prooxidant enzymes. More specifically, non-phagocytic NOX production may also be involved in the transformation of HSCs [27]. Another example is xanthine dehydrogenase, which is proteolytically cleaved to the $O_2^{\bullet-}$-producing xanthine oxidase in response to hypoxia and other stimuli. Indeed, the xanthine oxidase inhibitor, allopurinol, confers protection against alcohol-induced oxidative stress in hepatic and in extrahepatic tissues [28, 29].

Alcohol Interference with Antioxidant Defenses

In addition to its stimulation of prooxidant production, alcohol decreases antioxidant defenses. The depletion by ethanol of both cytosolic and mitochondrial energy supplies can indirectly impair cellular antioxidant defenses. There exists a host of proteins and systems involved in the

"antioxidant network." This family does not directly intercept prooxidants, but serve instead as ancillary reductants and maintain the catalytic activity of antioxidant proteins or small molecules. These reactions of course require cellular energy. Heavy alcohol consumption also causes nutritional deficiencies through decreased intake or malabsorption [30], e.g., of selenium, methionine, ascorbic acid, carotene, and tocopherol, that can impair antioxidant defenses [31].

Even under optimal conditions, ROS/RNS will damage biomolecules, which often impairs function. Alcohol often simultaneously impairs the systems in place to repair and/or recycle these damaged proteins. For example, alcohol consumption inhibits the 26S proteasome [32], which is critical for degrading oxidatively modified proteins. Another process that is impaired by alcohol consumption is autophagy [33], which blunts the cells' ability to clear components that are damaged by ROS/RNS [34]. Impairment of these degradation processes can lead to the accumulation of damaged proteins within the cell, which can subsequently exacerbate other stresses (e.g., ER stress).

Effects of Alcohol Consumption on Liver Fat Homeostasis

The presence of macrovesicular fat droplets (hepatic steatosis) reflects an imbalance between uptake, synthesis, and export of fat and is a marker of liver stress. Once considered benign, steatosis may lead to steatohepatitis, fibrosis, and ultimately cirrhosis. ALD may evolve directly from fatty liver to fibrosis without a clinically apparent episode of hepatitis, although it is impossible to exclude low-grade asymptomatic steatohepatitis, such as occurs in NASH. Thus, understanding the mechanisms by which fat accumulates in the liver and the underlying metabolic abnormalities it reflects is essential to the development of treatment strategies. Lipids may accumulate in the liver through a number of mechanisms. Increased storage of lipids by the liver may occur through increased availability from diet or from increased lipolysis of adipose tissue. Lipids may also accumulate in the liver through de novo synthesis or through impaired fatty acid oxidation. Finally, impaired export of triglycerides can lead to increased hepatic lipid content.

Hepatic Lipid Uptake

Dietary Intake

Most dietary fat is packaged and transported as chylomicrons and the fatty acids transferred for storage in the adipose tissue after hydrolysis of the fat by lipoprotein lipase; its transfer to the liver is discussed below. The fat content of the diet also modifies the development of fatty liver when alcohol is fed. Rats fed isocaloric diets in conjunction with ethanol demonstrated increased hepatic triglyceride content when greater than 25 % of the daily calories were from fat [35], the usual situation in humans. The type of fat in the diet can alter the degree of hepatic steatosis; for instance, alcohol-fed rats had more severe steatosis and liver injury when given a diet rich in polyunsaturated fatty acids (PUFA) compared with those given alcohol on the background of a diet high in saturated fats [36–38]. These effects may reflect several phenomena: the protective effect of saturated fats may result from less lipid peroxidation (see below), the increased release of adiponectin, an adipokine which is downregulated in ALD [38] that is seen with saturated fat intake, and the effects of dietary fat composition and quantity on gut permeability, and the resultant effect on the levels of LPS in the portal blood.

The Role of Adipose Tissue

Heavy alcohol use alters adipose tissue metabolism and contributes to the development of steatosis. Studies of body composition show reduced adipose mass with increased alcohol use [39–42]. Heavy drinkers had higher resting energy expenditure and lower nonprotein respiratory quotient, indicating greater rates of fatty acid oxidation. These changes were reversible with 3 months of

abstinence. Moderate drinking did not change metabolic rate or postprandial free fatty acid or insulin levels [43]. Similarly, chronic ethanol feeding of rats reduced adipose tissue (perirenal and epididymal fat) deposits by about 30 % compared with pair-fed controls [44].

Alcohol may modify intracellular metabolism and signaling pathways of adipocytes or alter circulating hormones, metabolites, or cytokines. Adipose tissue homogenates generated acetaldehyde from ethanol, and CYP2E1 has been detected in white adipose tissue of rats [45]. Ethanol treatment of rat adipocytes inhibited the conversion of glucose to glyceride-glycerol [44]. Chronic ethanol feeding elevates plasma levels of FFA and reduced adipose tissue glucose oxidation and conversion of glucose to triglyceride. Sebastian and Nagy [46] reported that chronic ethanol feeding leads to insulin resistance by disruption of the Cbi/TC10 pathway and impaired GLUT4-dependent glucose uptake in adipose cells. Chronic alcohol feeding reduces the ability of insulin to inhibit lipolysis in white adipose tissue, thus increasing the rate of triglyceride breakdown. Song reported that there was increased homocysteine and SAH, with decreased SAM and mRNA for cystathionine synthase, in fat from mice fed an ethanol-containing diet. Homocysteine induced an ER stress response in isolated adipocytes and in adipose tissue from ethanol-fed mice [47]. Alcohol feeding leads to elevated TNFα levels in fat tissue, with reduced adiponectin secretion [48]. Furthermore, alcohol feeding of mice downregulated adipose cell PPARγ and white fat mass [49], stimulated lipolysis, and reduced triglyceride synthesis: these effects were reversed with the PPARγ agonist rosiglitazone. These changes correlated with increased adiponectin levels and improved steatosis and inflammation in the liver. A similar study also showed beneficial effects of rosiglitazone on steatosis [50] that were associated with increased adiponectin levels and activation of sirtuin 1 (SIRT1) and AMP-activated protein kinase (AMPK) signaling which are known to block hepatic de novo lipid synthesis and increase fatty acid oxidation (see below).

Fatty Acid Transporters

Circulating fatty acids are transported into the hepatocytes via CD36, a fatty acid translocase [51]. Zhou et al. [52] demonstrated that induction of CD36, through pharmacologic or genetic activation of the nuclear receptor LXR (liver X receptor), led to hepatic steatosis and such effect can be prevented in CD36-null mice. In addition to CD36, two other regulators of fatty acid transport, fatty acid transport protein (FATP) and fatty acid-binding proteins (FABPs), have been demonstrated to play a possible role in hepatic steatosis. Deletion of these proteins reverses hepatic steatosis induced by diet in the mouse model [53–55]. The role of fatty acid transport has not been extensively studied in ALD. Alcohol feeding increases the level of CD36 [56] and FATP-2 [57]. Adiponectin treatment of ethanol-fed animals led to decreased levels of hepatic CD36 and prevented hepatic steatosis [58]; however, adiponectin has a number of other effects on fat metabolism.

Hepatic Lipid Synthesis

De novo lipid synthesis plays an important role in ALD. Carbohydrates are converted to triglycerides through breakdown to acetyl-CoA which then enters the fatty acid synthesis pathway [59], including ATP citrate lyase, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase (SCD1).

Activation of Steroid Response Element-Binding Protein-1c (SREBP-1c)

SREBPs are critical for the regulation of fatty acid, triglyceride, and cholesterol synthesis. In their inactive form, SREBPs are bound to the endoplasmic reticulum (ER) and nuclear envelope. Mature forms are released by proteolysis, processed in the Golgi, and then transported into the nucleus, where SREBP-1c activates genes involved in fatty acid synthesis, while SREBP-2 modulates genes of cholesterol synthesis [60, 61]. Overexpression of SREBP-1c in mice leads to the development of extensive hepatic steatosis [62] and has been implicated in the pathogenesis of ALD.

Active SREBP-1c and its battery of lipogenic genes are increased in alcohol-fed animals [63], and SREBP-1c knockout mice are protected from alcohol-induced hepatic steatosis [64].

Alcohol activates SREBP-1c by many mechanisms (Fig. 3.1). The endoplasmic reticulum (ER) stress response is triggered by the accumulation of unfolded/misfolded proteins, resulting in increased levels of proapoptotic proteins and induction of transcriptional SREBP-1c and SREBP-2 that are necessary for the repair of cell membranes. Mice fed high doses of alcohol had increased expression of GRP78 (glucose-regulated protein 78), GRP94, CHOP (C/EBP homology protein), and caspase 12, proapoptotic proteins in the ER stress response pathway [65]. These mice also demonstrated increased levels of active SREBP-1c. Thus, ER stress participates in both the steatosis of ALD and the sensitization of the cells to apoptosis (see below).

Alcohol induces ER stress through several mechanisms, including direct effects of acetaldehyde [66], oxidative stress [67], and elevated homocysteine. Homocysteine is elevated because alcohol interferes with the methionine cycle, whereby methionine is converted to S-adenosylmethionine (SAM) by methionine adenosyltransferase 1 (MAT1). SAM is converted to *S*-adenosylhomocysteine (SAH) by donating a methyl group to an accepting molecule, and SAH is hydrolyzed to homocysteine, which is converted to methionine by either methionine synthase (MS) or betaine-homocysteine methyltransferase (BHMT). Homocysteine is elevated in chronically ethanol-fed animals and in human alcoholics [68–71], and SAM is decreased, due to folate deficiency and to decreased activity of methionine synthase [72].

Mice fed homocysteine rapidly developed ER stress [67]; mice fed alcohol developed large increases in homocysteine; treatment with betaine, which acts as a methyl donor for BHMT, decreased levels of homocysteine and SREBP-1c [65]. In micropigs fed alcohol and a folate-deficient diet, SREBP-1c levels correlated with homocysteine levels [73], and this was reversed by administration of SAM, presumably through its inhibitory effects on ER stress [74]. Additionally, transgenic mice expressing human BHMT were resistant to alcohol-induced increases in homocysteine and hepatic steatosis [67].

SREBP-1c is also activated by acetaldehyde [63], as well as lipopolysaccharide (LPS) and TNFα, all of which are increased in ALD [75] even at very early stages. In fact binge drinking by normal volunteers increased circulating LPS [76], and acute exposure to ethanol increases SREBP-1c mRNA and activity [77]. Inhibition of AMPK, discussed below, increased stability and activity of SREBP-1c [78]. Thus, many effects of alcohol converge to activate SREBP-1c (Fig. 3.1).

Carbohydrate-Responsive Element-Binding Protein

Carbohydrate-responsive element-binding protein (ChREBP) is a glucose-responsive transcription factor [79], whose activity is regulated by nuclear/cytosolic partitioning and posttranslational modification. ChREBP induces transcription of genes involved in glycolysis [liver pyruvate kinase (LPK)], lipogenesis (ACC and FAS), and gluconeogenesis (glucose-6-phosphatase (G6Pase) [80]. ChREBP is inhibited by phosphorylation by protein kinase A and AMPK [81]. AMPK activity is sensitive to the energy state of the cell; under low energy conditions, AMPK is active, and ChREBP is retained in the cytosol. When glucose is abundant, AMPK is suppressed, leading to nuclear translocation of ChREBP and induction of glucose-responsive genes. ChREBP activity is activated by dephosphorylation. Specific isoforms of protein phosphatase 2A (PP2A) are activated by xylulose-5-phosphate (Xu-5-P), an intermediate in the pentose phosphate shunt. When glucose is high, it is converted to Xu-5-P, activating PP2A and ChREBP [82].

Alcohol infusion significantly increased hepatic mRNA and protein expression of ChREBP in rats [83]. Treatment of hepatoma cells with alcohol activated a ChREBP-dependent reporter, an effect blocked by ChREBP siRNA. Alcohol caused nuclear translocation of ChREBP and increased its ability to bind DNA, leading to an increase in the expression of the ChREBP gene battery [84]. Alcohol may activate ChREBP via

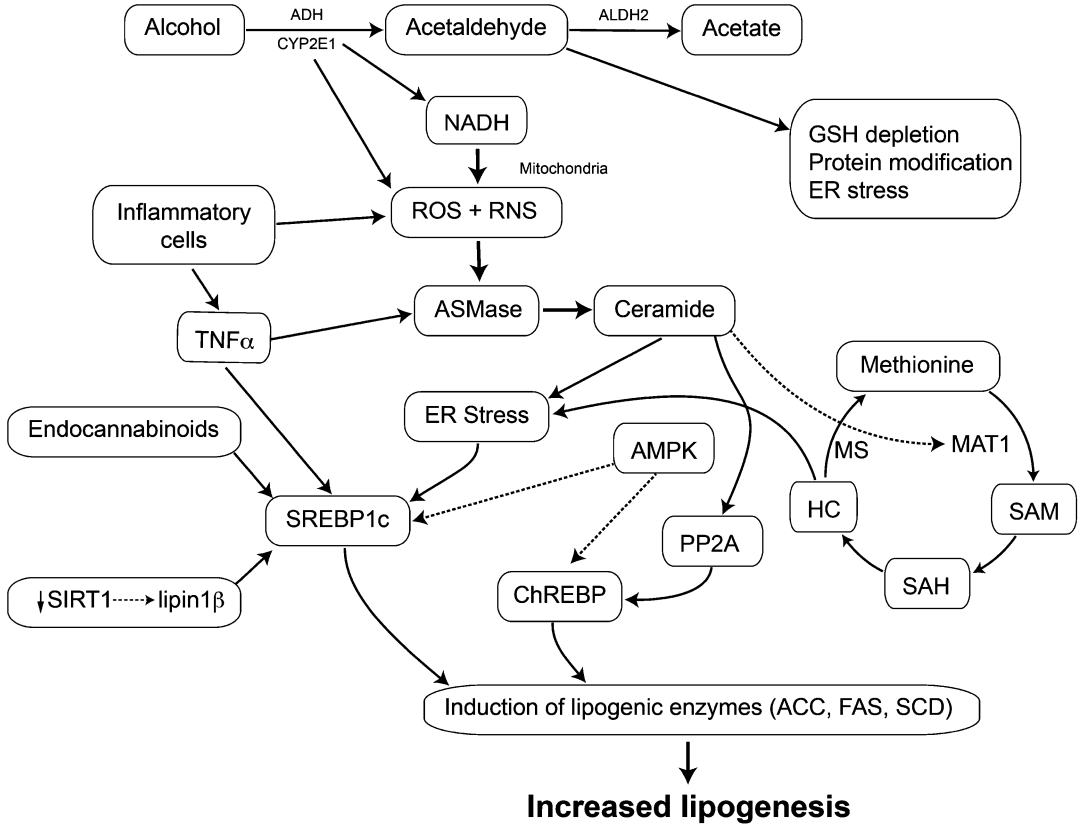

Fig. 3.1 Effects of heavy alcohol use on metabolic and signaling pathways leading to increased hepatic fat synthesis. Heavy alcohol use increases hepatic triglyceride content via increased uptake of fatty acids from the circulation and increased de novo synthesis. The increase in de novo synthesis is mediated by activation of transcription factors which induce the synthesis of a battery of enzymes involved in the conversion of acetyl-CoA to fatty acids. Abbreviations: *ADH* alcohol dehydrogenase, *ALDH2* aldehyde dehydrogenase 2, *SREBP-1c* steroid response element-binding protein-1c, *ChREBP* carbohydrate-response element-binding protein, *ER* endoplasmic reticulum, *SIRT1* sirtuin 1, *ASMase* acidic sphingomyelinase, *AMPK* AMP-activated protein kinase, *TNFα* tumor necrosis factor-α, *MAT1* methionine adenosyltransferase 1, *MS* methionine synthase, *ACC* acetyl-CoA carboxylase, *FAS* fatty acid synthase, *SCD* stearoyl-CoA desaturase, *GSH* reduced glutathione, *ROS* reactive oxygen species, *RNS* reactive nitrogen species

activation of PP2A and inhibition of AMPK, but not by an increase in hepatic Xu-5-P (see below and Fig. 3.1). Induction of SREBP and ChREBP by alcohol synergistically increases lipogenesis.

Liver X Receptor (LXR) and Farnesoid X Receptor

LXR is an orphan nuclear receptor [85] activated by glucose, glucose-6-phosphate, and oxysterols that is involved in cholesterol homeostasis [86–89]. LXR regulates lipogenic genes such as FAS and ACC, as well as SREBP-1c and ChREBP [90–92]. LXR agonists induced CD36, the liver fatty acid uptake transporter, and an interaction between PPARα and SREBP-1 may be mediated by LXR [93]. Farnesoid X receptor (FXR) is the bile salt receptor which is the target of agonist drugs in clinical trials for nonalcoholic fatty liver and cholestatic liver diseases. FXR activity is reduced in alcohol-fed mice [94]. FXR agonists reversed the inhibition of FXR activity, suppressed induction of CYP2E1, reduced oxidative stress, reduced SREBP-1c activity, and reduced steatosis in animal models of ALD [95].

Ceramide Signaling and the Role of Acidic Sphingomyelinase (ASMase)

Activation of SREBP-1c and ChREBP may be coordinated by the signaling lipid ceramide. Ceramide is generated either de novo from serine and palmitoyl-CoA, via serine palmitoyltransferase (SPT), or from the breakdown of sphingomyelin (mediated by sphingomyelinases: acidic sphingomyelinase (ASMase) and neutral sphingomyelinase (NSMase)). Ceramide is further metabolized by ceramidase to sphingosine, which can be converted back to ceramide by (dihydro) ceramide synthase. Ceramide levels are increased by alcohol (see below). Most recent interest has centered on the ASMase pathway. ASMase is activated by reactive oxygen species and by TNFα, and ASMase mRNA is increased in biopsies from individuals suffering from alcoholic hepatitis [96]. When ASMase activity is reduced (by genetic knockout or by use of inhibitors), alcohol-induced lipogenesis is reduced; there is less cholesterol accumulation in the mitochondrion, less steatosis, and reduced sensitivity to LPS [96]. In ASMase knockout mice, alcohol does not induce ER stress, despite elevated levels of homocysteine, and homocysteine administration no longer induced ER stress. Other effects of ceramide deduced from experiments with ASMase knockout animals include reduction in MAT1A mRNA and activity; inhibition of liver CTP-phosphocholine cytidylyltransferase (CCT), which participates in phosphatidylcholine synthesis; and activation of ER stress triggered by inhibition of autophagy [96]. As described below, ceramide may have effects on AMPK as well. Thus, strategies aimed at blocking ASMase activity and reducing ceramide levels seem like a promising direction of therapeutic research (Fig. 3.1).

Role of Endogenous Cannabinoids

The endocannabinoid system has been studied for its role in fatty liver diseases [97]. Liver cells express low levels of cannabinoid (CB1) receptors. Alcohol feeding increases the production of the endogenous cannabinoid 2-arachidonoylglycerol (2-AG) by HSCs, which could interact with hepatocytes in a paracrine fashion. Alcoholic steatosis was attenuated with CB1 antagonists, and CB1 knockout animals are resistant to alcoholic fatty liver [98]. In the knockout animals or animals treated with CB1 antagonists, the levels of SREBP-1c and FAS were reduced relative to control alcohol-fed animals, while CPT1 activity is increased. Activation of CB1 receptors increased the ability of LXR to induce SREBP-1c, and blockade of CB1 receptors increased protein kinase A and AMPK activity, inhibiting LXR action [99]. Thus, endocannabinoids, and by extension use of marihuana, may aggravate ALD through induction of SREBP and inhibition of AMPK.

Hepatic Fatty Acid Oxidation

Decreased fatty acid oxidation can cause hepatic steatosis. Impaired fatty acid oxidation plays an important role in ALD and is mediated by actions of alcohol on energy sensors and transcription factors.

AMP-Activated Protein Kinase (AMPK)

AMPK is a serine/threonine kinase which regulates energy homeostasis by stimulating energy-producing processes, e.g., fatty acid oxidation and glycolysis, and inhibiting energy-utilizing processes, e.g., fatty acid and amino acid synthesis. This occurs, in part, through phosphorylation and inactivation of ACC, decreasing the levels of malonyl-CoA, a precursor in the synthesis of fatty acids and an inhibitor of carnitine palmitoyltransferase 1 (CPT1). CPT1 is the rate-limiting enzyme in fatty acid oxidation. Thus, AMPK is able to exert its effects on both fatty acid synthesis and oxidation through its effects on ACC [100–102]. AMPK also inhibits SREBP-1c and ChREBP activity [78, 81, 103], reducing de novo lipogenesis.

AMPK activity is decreased in alcohol-treated hepatoma cells and in the liver, with a concomitant increase in ACC activity and steatosis [78, 104]. Activation of AMPK with metformin blocks the effect of alcohol on ACC activity, SREBP-1c, and reduces steatosis [78]. Control of AMPK activity is mediated by phosphorylation on a

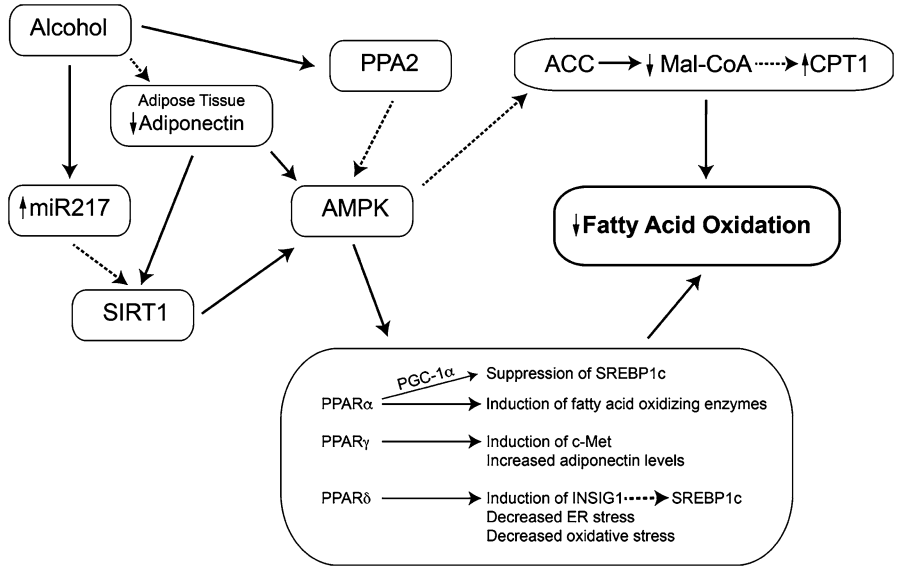

Fig. 3.2 Effects of heavy alcohol use on the controls of fatty acid oxidation. Heavy alcohol use reduces the rate of fatty acid oxidation in the liver. This is mediated in particular by inhibition of AMP-activated protein kinase (AMPK) (which modifies both the activity of SREBP-1c and the entry of fatty acyl-CoA esters into the mitochondrion) and peroxisome proliferator-activated receptors. As a result, fatty acids are stored as triglycerides. Abbreviations: *SIRT1* sirtuin 1, *AMPK* AMP-activated protein kinase, *PP2A* protein phosphatase 2A, *PPAR* peroxisome proliferator-activated receptor, *FFA* free fatty acids, *Mal-CoA* malonyl-CoA, *PGC-1* PPARγ coactivator-1, *CPT1* carnitine palmitoyltransferase 1

threonine residue at position 172; three main mechanisms for the effect of alcohol on AMPK have been reported (Fig. 3.2).

Regulation of AMPK by PP2A and Ceramide

Protein phosphatase 2A (PP2A) is a serine/threonine phosphatase regulating cellular functions and signaling pathways, including ChREBP, apoptosis, and insulin signaling [105], and may be involved in alcohol inhibition of AMPK. A PP2A subunit co-immunoprecipitated with AMPK, alcohol increased PP2A activity in hepatoma cells [106], and the PP2A inhibitor okadaic acid or PP2A siRNA significantly attenuated the inhibitory effect of alcohol on AMPK phosphorylation.

PP2A is regulated by ceramide [107]; in fact, a form of PP2A was originally named the ceramide-activated protein phosphatase [108]. Alcohol treatment of hepatoma cells significantly increased cellular (C16 and C18) ceramide content and increased PP2A activity [106]. Inhibitors of the de novo synthetic pathway and NSMase did not prevent inhibition of AMPK by alcohol; however, the ASMase inhibitor imipramine did prevent this effect. This effect of alcohol was observed in vivo: in mice fed alcohol, there were 30 % increases in hepatic C16 and C18 ceramides, the level of ASMase mRNA was increased by 1.7-fold, and imipramine reduced ASMase activity, ceramide levels, PP2A activity, and triglyceride accumulation [109]. The increase in ASMase activity was not associated with increased levels of TNFα, and imipramine treatment did not restore AMPK activity. More recently, myriocin, an inhibitor of de novo ceramide synthesis, reversed alcoholic steatohepatitis in animals [110].

Alcohol-induced elevation of ceramide may contribute to the induction of steatosis by AMPK-dependent and AMPK-independent pathways.

Regulation of AMPK by Adiponectin

Adiponectin stimulates fatty acid oxidation, in part through its activation of PPARα and

AMPK [38]. Alcohol feeding decreased plasma adiponectin levels [38, 47, 111] and the adiponectin receptors AdipoR2 and AdipoR1 in mice and micropigs, respectively [74, 111]. Treatment of alcohol-fed animals with adiponectin alleviated hepatic steatosis [58]. Conversely, adiponectin knockout mice are highly sensitive to alcohol-induced steatosis, but surprisingly no difference in AMPK activity or PPARα DNA-binding activity was observed [112]. Interestingly, adiponectin can reduce ceramide levels, suggesting another mechanism by which it improves liver metabolism in alcohol-fed animals.

Regulation of AMPK and Lipid Metabolism by SIRT1

SIRT1 is an NAD^+-dependent protein deacetylase which senses the cellular redox state as a measure of energy need and can regulate the activity of proteins involved in lipid metabolism. SIRT1 is as a major regulator of AMPK [113]. Liver-specific knockout of SIRT1 results in hepatic steatosis and inflammation [114]. Alcohol feeding and in vitro studies show that metabolism of alcohol reduces SIRT1 activity [115, 116] and mRNA [117]. Mechanisms accounting for this effect include increased levels of miR217 in alcohol-metabolizing cells or liver [118]. This effect could be overcome with resveratrol, which also reversed steatosis [111], or feeding a high-saturated fat diet [115, 116]. Resveratrol increased circulating adiponectin and the activity of SIRT1, AMPK, and PPARγ coactivator-1α (PGC-1α) [111]. Reduction of SIRT1 activity has several downstream effects. Reduced SIRT1 activity leads to increased activity of SREBP-1c and changes the expression of lipin-1 (an Mg^{++}-dependent phosphatidic acid phosphohydrolase). This protein is a regulator of fatty acid oxidation and de novo lipogenesis [119]. Alcohol feeding, via SIRT1 inhibition, increases the level of lipin1β, which in turn led to the activation of SREBP-1c. SIRT1 was also reduced in macrophages exposed to acetaldehyde or acetate, and they generated increased amounts of TNFα [120]. Feeding alcohol to SIRT1 knockout animals increased markers of inflammation, oxidative stress, ER stress, cytokine production, and fibrosis, suggesting an important role for SIRT1 in modulating all these effects of alcohol. In samples of liver from patients with alcoholic hepatitis, SIRT1 mRNA was reduced to about 25 % of normal, although the effects on lipin mRNA were more subtle [114]. The effect of alcohol on lipin expression was blocked by activators of AMPK or constitutively active AMPK genes. Thus, SIRT1 (and, by extension, dietary polyphenols such as resveratrol) plays important roles in regulating fat synthesis and oxidation in ALD.

Peroxisome Proliferator-Activated Receptors

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that regulate genes involved in fatty acid oxidation and transport, lipid storage in adipocytes, and inflammation. Alcohol affects the activity of each of the three classes of PPARs.

Alcohol decreased the DNA-binding ability of PPARα, downregulating numerous PPAR-regulated genes [121]. Knockout of PPARα increased liver damage caused by alcohol feeding, including increased hepatomegaly [122]. PPARα inhibits the expression of SREBP-1c; hence, the reduction in PPARα activity by alcohol may be another mechanism of SREBP-1 induction. Pharmacological activation of PPARα prevents alcohol-induced fatty liver [123, 124]. Alcohol effects may involve AMPK, as the kinase may regulate PPARα through protein-protein interactions [125]. The action of PPARα depends on PGC-1α, which is activated by AMPK [126]. Alcohol feeding reduced the level of PGC-1α, and this was restored by changing the lipid in the diet to medium-chain triglyceride [117].

PPARγ is highly expressed in adipocytes, with low levels in the liver. It coordinates adipocyte differentiation, fatty acid uptake, and glucose metabolism. Based on pharmacological studies, PPARγ may play a role in ALD. Alcohol inhibited PPARγ transcriptional activity in cultured hepatocytes, but has also been shown to increase PPARγ mRNA [111, 127]. Treatment with the PPARγ agonist pioglitazone prevented the development of alcohol-induced steatosis [128]; however, pioglitazone also

activates AMPK [129]. Pioglitazone was also reported to prevent alcoholic fatty liver through the upregulation of c-Met [130], an activator of lipid export (below).

PPARδ modulates energy metabolism and, like PPARα, inhibits SREBP-1c, possibly via induction of insulin-induced gene 1 (INSIG1), an inhibitor of SREBP-1c maturation [131]. PPARδ knockout animals had greater accumulation of triglyceride during alcohol feeding, increased SREBP-1c levels [132], and increased CYP2E1 mRNA. Furthermore, PPARδ agonists reduced alcohol-induced insulin resistance and oxidative stress [133], as well as ER stress and ceramide generation [134]. In a comprehensive comparison, activation of each class of PPARs ameliorated alcoholic steatosis [135].

The Role of Autophagy in the Regulation of Hepatic Lipid Stores

Autophagy is a process whereby intracellular organelles and membranes are engulfed in a particle called the autophagosome, which then fuses with lysosomes to form autolysosomes. This process prolongs cell survival during nutrient starvation: the breakdown of intracellular materials, including lipids, provides energy as a temporizing measure. A larger role for autophagy in lipid homeostasis is suggested by the finding that inhibition of autophagy leads to hepatic steatosis (as shown with the *Atg7* knockout model). When autophagy is inhibited, the ER stress response is activated.

Studies on the effect of alcohol on autophagy show that acute alcohol gavage and chronic alcohol feeding increase the number of autophagosomes (estimated from the expression of the LC3II protein which is associated with these organelles). This appears to be dependent on alcohol metabolism and probably oxidative stress. It results from the inhibition of mTOR activity – the exact mechanism of this effect is unknown and is somewhat unexpected given that AMPK activation also leads to reduced mTOR activity and AMPK is decreased with alcohol feeding. A separate pathway regulating mTOR may thus play a role. It is not clear whether degradation of the contents of the autophagosome is altered in alcohol-fed animals: inhibition of fusion with lysosomes could contribute to the increase in autophagosomes. Autophagy may be a protective response to alcohol exposure, as suppression of autophagy made experimental alcoholic liver injury worse [136]. Autophagy may also serve to remove mitochondria damaged by heavy alcohol exposure [12]. The current state of this research, and the differences between acute and chronic exposure to alcohol, was recently reviewed [137].

Export of Triglycerides as Very Low-Density Lipoprotein

The liver packages triglycerides (TGs) into very low-density lipoprotein (VLDL) particles through the fusion of lipid droplets with apolipoprotein B100 (apoB100), which is dependent on the activity of microsomal triglyceride transfer protein (MTP). VLDL export is impaired in ALD through several mechanisms. Phosphatidylcholine (PC) is required for VLDL secretion, and the hepatic levels of this lipid are decreased with alcohol feeding. Two pathways produce PC: the majority is produced from choline, first phosphorylated and then converted to CDP-choline by CTP-phosphocholine cytidylyltransferase (CCT). CDP-choline then forms phosphatidylcholine by condensation with diacylglycerol. CCT is inhibited in alcohol-fed liver. Phosphatidylethanolamine methyltransferase (PEMT) produces perhaps 20–30 % of the needed PC by methylating phosphatidylethanolamine [138]. Inhibition of PEMT activity decreases PC levels and leads to steatosis [138, 139]. Since PEMT is a SAM-dependent enzyme, the low SAM levels in ALD leads to reduced PEMT activity [140, 141]. BHMT, which is decreased in ALD, can increase the expression of apoB100, probably via decreased levels of homocysteine [65, 142]. PPARα regulates the production of MTP; MTP levels were decreased in livers of alcohol-fed animals, while treatment with a PPARα agonist upregulated MTP and increased

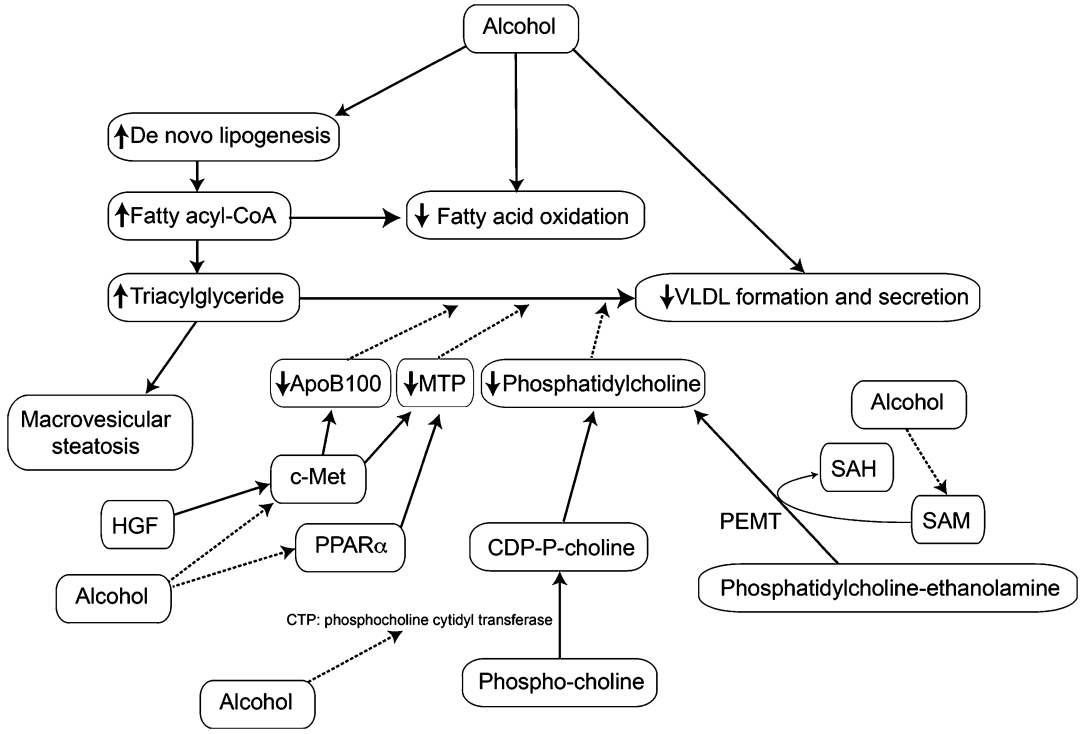

Fig. 3.3 Effects of heavy alcohol use on the export of triglyceride as VLDL. Heavy alcohol use increases the synthesis of fatty acids and triglycerides in the liver which are packaged as VLDL for export; however, alcohol use impairs this process below the rate needed to export all the excess triglyceride so that there may be both elevated VLDL in the circulation (hypertriglyceridemia) and hepatic steatosis. Abbreviations: *VLDL* very low-density lipoprotein, *HGF* hepatocyte growth factor, *c-Met* receptor for hepatocyte growth factor, *MTP* microsomal triglyceride transfer protein, *apoB100* apolipoprotein B100, *SAM* S-adenosylmethionine, *SAH* S-adenosylhomocysteine, *PEMT* phosphatidylethanolamine methyltransferase

export of VLDL [123, 143, 144]. Hepatocyte growth factor (HGF), acting though its receptor, encoded by the c-Met oncogene, stimulates the synthesis of apoB100 and MTP; although HGF levels are increased in alcohol-fed liver, c-Met levels are decreased [144]. Pharmacological administration of HGF overcomes this reduction in receptor level and reverses alcoholic steatosis [145]. Thus, impaired TG export contributes to ALD (Fig. 3.3). The potential mechanisms of steatosis in ALD are summarized in Table 3.1.

Inflammation and Cell Death in ALD

Fatty liver alone does not contribute to morbidity in alcoholic patients; as in nonalcoholic fatty liver, the development of inflammation, cell death, and ultimately fibrosis are much more important. Oxidant stress, endotoxemia, innate immunity, and apoptosis contribute to these processes.

Inflammation

Alcohol exposure impacts the inflammatory/immune response via a myriad of mechanisms and can either tolerize or sensitize the liver to a second inflammatory stimulus [146]. The effect of alcohol may be determined by the relative timing of the two responses. For example, if lipopolysaccharide (LPS) is administered during acute alcohol intoxication, the alcohol exposure blunts hepatic inflammation and injury caused by the LPS; in contrast, if LPS is given ~1 day after alcohol exposure or after chronic alcohol

Table 3.1 Mechanisms by which alcohol use causes steatosis

Pathways	Mechanism	Consequences
Increased FFA flux from adipose tissue	Reduced insulin sensitivity of adipose; adipose tissue inflammation	Elevated FFA delivered to the liver for storage
Reduced adiponectin	Direct effect of alcohol on adipose	Reduced AMPK activity, reduced FFA oxidation
Inhibition of methionine cycle	Inhibition of MAT1, MS: decreased SAM, increased homocysteine	ER stress, reduction in phosphatidylcholine synthesis and other methylation reactions
ER stress	Oxidative stress, increased ceramide, increased homocysteine	Proapoptotic, increases SREBP-1c
Altered miRNA levels	Unknown	Decreased SIRT1, increased intestinal permeability, sensitization of Kupffer cells
Increased ceramide	Activation of ASMase by TNF, ER stress	Activation of PP2A, inhibition of phosphocholine synthesis, inhibition of AMPK, inhibition of MAT1
Inhibition of AMPK	Reduced SIRT1, increased ceramide, increased PP2A	Reduced fatty acid oxidation, reduced de novo fat synthesis
Increased SREBP-1c activity	Reduced AMPK, reduced SIRT1, ER stress	Increased de novo fat synthesis
Increased ChREBP activity	Decreased AMPK, increased PP2A	Increased de novo fat synthesis
Reduced c-Met expression	Increased ceramide	Reduced MTP and apoB100 expression, decreased VLDL export
Increased endocannabinoids	Increased SREBP-1c	Increased de novo fat synthesis
Decreased PPAR activity	Acetaldehyde, decreased AMPK activity	Increased SREBP-1c, decreased c-Met, decreased fatty acid oxidation

Abbreviations: *FFA* free fatty acids, *SREBP-1c* steroid response element-binding protein-1c, *ChREBP* carbohydrate-response element-binding protein, *ER* endoplasmic reticulum, *ASMase* acidic sphingomyelinase, *AMPK* AMP-activated protein kinase, *TNF* tumor necrosis factor, *SIRT1* sirtuin 1, *PP2A* protein phosphatase 2A, *c-Met* receptor for hepatocyte growth factor, *MTP* microsomal triglyceride transfer protein, *apoB100* apolipoprotein B100, *PPAR* peroxisome proliferator-activated receptor, *MAT1* methionine adenosyltransferase 1, *MS* methionine synthase

exposure, the inflammatory response is synergized. In general, however, it can be said that alcohol exposure enhances normal inflammatory tissue damage caused predominantly by the innate immune response.

Alcohol-Induced Endotoxemia

The strategic location of the liver between the intestinal tract and the rest of the body makes it a critical organ for clearance of xenobiotics and toxins that enter the portal blood. It is therefore not surprising that the liver has a very high capacity for phase I and II metabolic processes, which is responsible for the well-known "first-pass effect" in xenobiotic metabolism. As early as 1893, Pavlov observed that low levels of intestinally derived "toxins" are normally present in the portal blood and are cleared by the liver [147]. Kupffer cells, the resident hepatic macrophages, are responsible for clearing endotoxin [148] (Fig. 3.4). When high levels of LPS are present in the portal blood, Kupffer cells are activated and can mediate toxic responses in the liver [149]. It has been known since the 1970s that alcohol consumption increases the amount of detectable LPS in the blood [150, 151]. These increases are thought to be caused by both dysbiosis in the GI tract microbiome and a decrease in GI tract barrier function. Importantly, these are highly interdependent functions in that dysbiosis decreases GI tract barrier and altered barrier function can impact the microbiome.

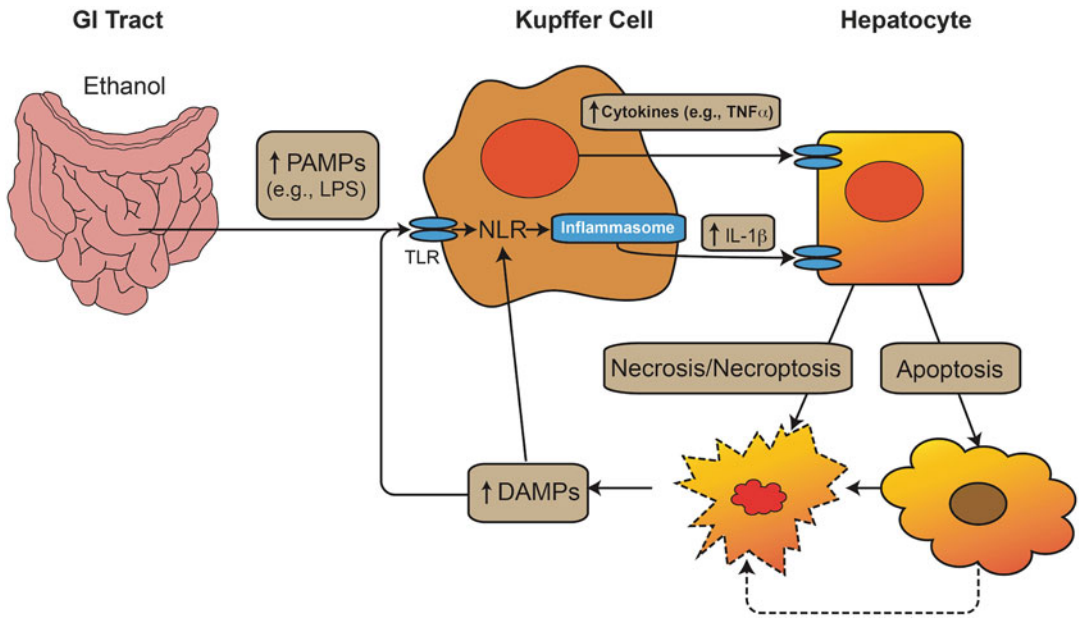

Fig. 3.4 Interaction between danger signals and the innate immune response in ALD. Alcohol increases gut permeability and increases the level of pathogen-associated molecular pattern molecules (PAMPs), such as lipopolysaccharide (LPS) in the portal blood. These PAMPs interact with toll-like receptors (TLR) to increase de novo synthesis of proinflammatory cytokines (e.g., TNFα). These signals also activate the inflammasome complex via NOD-like receptors (NLRs) to proteolytically activate IL-1β. These cytokines induce cell death via necrosis or apoptosis; furthermore, the energetic stress on the cells caused by alcohol exposure can lead to necrosis secondary to the initiation of apoptosis (i.e., necroptosis). Products of hepatocyte stress and death can serve as damage-associated molecular pattern (DAMP) molecules, which can also stimulate macrophages, either via TLR signaling or via direct activation of NLRs and the inflammasome. The result is a vicious cycle of inflammation and cellular injury. Alcohol enhances the inflammatory response of innate immune cells (i.e., priming effect) and exacerbates the cytotoxic effect of cytokines on parenchymal cells (i.e., sensitizing effect)

Dysbiosis

The concept that the microbiome can contribute to liver disease was first shown experimentally more than 60 years ago when nonabsorbable antibiotics protected against choline-deficiency-induced liver injury [152]. Today, the gut-liver axis, and enteric dysbiosis, is a generally accepted component in the development of liver disease. Indeed, ALD was a pioneering disease in which dysbiosis was considered a mechanism of tissue injury and commensal bacterial supplementation was demonstrated to protect against experimental ALD over 20 years ago [153].

The advent of platform-based analysis approaches (e.g., deep sequencing) and bioinformatics approaches has dramatically increased our understanding of how rapidly and dynamically the microbiome can change and affect change in ALD [154, 155]. These studies have validated early studies that indicated that alcohol exposure expands the population of Gram-negative bacteria in the gut, which explains, in part, the low-grade endotoxemia caused by alcohol. However, these studies, coupled with functional analyses, such as metabolomic profiling, have identified a much more active role of the microbiome in maintaining liver health. Indeed, recent work indicates that protective effects mediated by diet (e.g., saturated fat [156]) or by knocking out key genes (e.g., TNFR1 [157]) may have as much to do with the response of the microbiome to these changes as to the host's [158, 159]. This rapidly advancing field will likely continue to yield new insight into ALD for the foreseeable future.

Decreased Gut Barrier Function

The ability of the mucosal barrier to withhold macromolecules is imperfect, and small amounts of antigens and other products normally traverse the intestinal wall. To a certain extent, this "leakiness" of the GI tract is important for both new antigen presentation and development of immune tolerance. However, if the barrier is significantly impaired, the innate immune response can be activated and can thereby induce a local and/or systemic inflammatory response. Alcohol has been known to increase the permeability of the GI tract to small molecules [160]. The barrier function of the GI tract is multilayered and includes physical barriers, such as the epithelial cells and the apical junctional proteins, as well as an active and robust immune response. Alcohol appears to decrease the barrier function via several of these pathways. Alcohol exposure directly damages epithelial cells and may cause GI tract erosions [160]. Alcohol also decreases the surface expression of apical junctional proteins (e.g., ZO-1), which are critical for maintaining normal tight junction barriers [161, 162]. Alcohol also induces a localized inflammatory response that again likely contributes to GI tract damage and decreased barrier function [155, 163]. Whereas the totality of the effect of ethanol on GI tract barrier function is incompletely understood, it is now well established that the barrier function is impaired and that the increase in permeability likely contributes to the initiation and development of ALD.

Priming of the Innate Immune System in ALD

The concept of priming and sensitization is important in ALD. Activation of the inflammatory response is, at least in part, due to increased levels of LPS [164, 165]. However, elevated levels of LPS found in alcoholics and in experimental ALD are relatively low compared to those found in experimental endotoxemia and human sepsis. Furthermore, liver injury caused by alcohol cannot be mimicked by chronic low-dose LPS in the absence of ethanol [166]. This is explained by the fact that inflammatory cells are primed to activation by alcohol administration, e.g., peripheral blood monocytes from patients with alcoholic hepatitis spontaneously produce proinflammatory mediators (e.g., TNFα). In response to LPS, these monocytes produce more proinflammatory mediators than their control counterparts [167]. Hepatocytes also appear to be sensitized to inflammatory stimuli by alcohol administration. Although TNFα is proproliferative in hepatocytes isolated from naïve animals, it is proapoptotic in cells isolated from alcohol-treated animals [168], via cellular "death domain" pathways [169]. HSCs also are sensitized by alcohol administration [170, 171]. The concept of priming and sensitization also means that there may be a series of sequential events in the progression of liver damage due to alcohol exposure. Specifically, the priming of inflammatory cells by alcohol leads to greater cell injury of sensitized hepatocytes; hence, blocking the activation of Kupffer cells [172] or employing knockouts with impaired Kupffer cell responses [173] prevents hepatocyte damage.

The enhanced production of TNFα is considered a key factor in the priming effect of alcohol on macrophage activation by LPS. Alcohol causes changes in the signaling cascade from the LPS receptor partners (CD14/TLR4) to the expression of TNFα. The expression of CD14 on Kupffer cells is upregulated by oxidant-dependent activation of the transcription factor AP-1 by alcohol [174]. Two transcription factors that are critical in TNFα expression are NFκB and EGR-1. Alcohol increases NFκB activation via both ROS-dependent [175] and ROS-independent pathways [176]. Ethanol also increases EGR-1 transcriptional activity via enhancing ERK1/ERK2 signaling [177]. Furthermore, alcohol itself enhances LPS-stimulated ROS production by macrophages via MyD88-independent TLR4 signaling [178]. Alcohol-induced TNFα expression is also regulated by increased mRNA stability. LPS stimulation of p38 mitogen-activated protein kinase contributes to this stabilization of TNFα mRNA. Moreover, studies have shown that at least one mRNA-binding protein, HuR, is also involved in the stabilization of TNFα mRNA stability after chronic exposure to ethanol (see [179] for review). The net effect is an increase in

TNFα protein release in alcohol-exposed macrophages after stimulation.

The sensitization effect of alcohol on hepatocytes represents an increased sensitivity of parenchymal cells to cytotoxic killing. TNFα-induced signaling via TNFR1 appears to play a key role. In normal hepatocytes, TNFα is a mitogen; however, alcohol causes changes in intracellular signaling that switch this mitogenic response to a cytotoxic response. There is a critical role of CYP2E1 induction in this process [180]. Using the model of CYP2E1 induction by pyrazole, they have shown that sensitization was reduced by half in CYP2E1 knockout mice. Sensitization was associated with the activation of p38 and JNK kinases, and liver injury was blocked with inhibitors of these kinases. Liver mitochondria from animals treated with pyrazole and TNFα underwent increased swelling in response to calcium, and cyclosporine A, an inhibitor of the mitochondrial permeability transition, reduced liver injury. Pyrazole increased the levels of IkB, which reduces the activation of NFκB by TNFα. NFκB is thought to mediate cell-protective effects of TNFα; this inhibition would tilt the effect of TNFα toward cell injury. The sustained activation of JNK (which is an apoptotic signal) coupled with the inhibition of NFκB activation are proposed to mediate the sensitization effect of alcohol (see [181] for review). Administration of N-acetylcysteine to mice treated with pyrazole and LPS or TNFα ameliorated in the changes in p38, JNK, and activation of NFκB. Further, pyrazole plus TNFα administration caused less liver injury in NOS2 (iNOS) knockout animals than in controls, indicating that peroxynitrite radicals contributed to sensitization of the liver. Thus, there is strong evidence from animal studies that oxidative stress, in particular that resulting from induction of CYP2E1, is very important in response of the liver to increased levels of LPS and TNFα.

MicroRNA in ALD

MicroRNAs are ubiquitous regulators of gene expression, which interact with mRNAs and can either block translation or stabilize the mRNA. Levels of a host of miRNAs have been reported to be altered in alcohol-fed animals or in cultured cells (reviewed by McDaniel et al. [182]). Some miRNAs are increased (miR21, miR34a, miR155, and miR320) and some are decreased (miR122, miR181a, miR199a, and miR200a) by alcohol exposure. Recent studies have indicated that these miRNAs are likely involved in many of the pathological responses to alcohol already discussed, including the modulation of SIRT1 (miR34a) and of intestinal permeability (miR122), apoptosis [183], and the sensitization of Kupffer cells to LPS (miR155). These RNAs may be targetable for therapy of ALD and may also provide serum biomarkers for different stages of the disease.

Contribution of Cell Death to Inflammation

As mentioned above, the strategic location of the liver between the intestinal tract and the rest of the body makes it a critical organ for clearance of xenobiotics and toxins that enter the portal blood; as such, the liver has a high likelihood of toxic injury. Hepatocellular death, when controlled, may clear damaged or dysplastic cells and thereby protect the organism as a whole; however, uncontrolled or high levels of cell death can induce toxic inflammatory responses.

Apoptosis, Necrosis, and Necroptosis

The two major death responses available to the cell are necrosis and apoptosis (Fig. 3.4). Necrosis is generally an acute response to traumatic stress and is characterized by frank autolysis of the cell and, in its most severe form, is independent of any intracellular signaling process. Apoptosis is a controlled ATP-dependent response to intrinsic and/or extrinsic stimuli. In contrast to necrosis, cellular fragments are released as blebs, and there is no release of intracellular contents into the extracellular space. As will be discussed below, necrosis is considered more proinflammatory by inducing the inflammasome. Both apoptosis and necrosis are validated histologies in fatty liver diseases, including ALD. However, the relative contribution of apoptosis histologically to ALD

is lower than would be expected by in vitro studies or in studies with inhibitors of apoptosis (e.g., [184]). It is now understood that apoptosis and necrosis are not distinct events, but rather part of a continuum of cellular responses to cytopathic stress. In this, continuum may extend to very early effects of alcohol, such as ER stress. ER stress was reported to stimulate the association of IRF3 with an adaptor protein (stimulator of interferon genes or STING) and the activation of IRF3, which is proapoptotic. This effect was independent of the development of inflammation [185]. An apoptotic response may convert to necrosis, if cellular energy stores are insufficient to complete the apoptotic process (i.e., "necroptosis") [186]. The metabolic stress caused by alcohol exposure that is described above (Sections "Metabolism of Ethanol and Its Role in Oxidative Stress" and "Effects of Alcohol Consumption on Liver Fat Homeostasis") may thereby cause necroptosis [187].

Stimulation of the Inflammasome by Cell Death

Inflammation is induced not only by pathogen-associated molecular patterns ("PAMPs"), such as LPS (see section "Contribution of cell death to inflammation"), but also by molecules released from dead or dying cells (damage-associated molecular patterns (DAMPs)) (Fig. 3.4). These danger signals interact with the toll-like receptor (TLR) family of pattern recognition receptors, which thereby activate intracellular sensor molecules, the NOD-like receptors (NLRs), to subsequently activate caspases. The net result is the proteolytic activation and release of IL-1β, which induces the innate immune response [188]. As apoptosis does not release intracellular contents into the extracellular space, it is assumed to be less likely to induce the inflammasome response. However, it should be emphasized that apoptosis is not completely benign. Apoptotic bodies have been shown to activate the innate immune response, as well as to stimulate HSCs in the liver [189]. Another emerging area of intercellular communication in response to stress is the release of exosomes. Exosomes are small vesicles of extracellular membrane released by cells (e.g., hepatocytes) in response to stimuli. These exosomes can serve as DAMPs to stimulate the inflammasome response [190].

Plasminogen Activator Inhibitor-1 (PAI-1) and Its Potential Role in ALD

PAI-1 is an acute phase protein that is typically expressed in adipocytes and endothelial cells, but it can be induced in response to stress [191]. The classical role of PAI-1 is to inhibit the plasminogen activators, tPA and uPA, thus blocking activation of plasminogen to plasmin and fibrinolysis. A number of studies also implicate PAI-1 in ALD.

Acute and chronic alcohol-induced steatosis is prevented by genetic or pharmacologic inhibition of PAI-1 expression [192]. Plasminogen activators cleave latent hepatocyte growth factor (HGF) to the mature active form [193, 194], which is important in lipoprotein secretion [195] (see section "Export of Triglycerides as Very Low-Density Lipoprotein"). Indeed, genetic or pharmacologic inhibition of PAI-1 expression caused an increase in c-Met phosphorylation, apoB100 expression, as well as VLDL secretion after alcohol exposure [192]. These data suggest that the protective effect of PAI-1 inhibition is due to a compensatory increase in VLDL synthesis.

In addition to blocking steatosis, preventing PAI-1 induction completely protected against chronic alcohol-induced inflammation [192]. The "classical" role of PAI-1 in impairing fibrinolysis may contribute to inflammation (see below). Furthermore, PAI-1 may alter the profile of other inflammatory mediators via inhibition of plasminogen activators independent of fibrin. For example, the inhibition of plasmin activation by PAI-1 prevents the conversion of secreted latent TGFβ to its active form [196], which may mediate anti-inflammatory effects, especially on monocytes/macrophages [197]. These potential mechanisms are not mutually exclusive and may occur in tandem [198].

Extracellular Matrix (ECM) Changes During ALD

The role of the ECM is well established in the setting of chronic liver injury, such as ALD. Indeed, robust changes in the hepatic ECM are the hallmark of later stages of liver disease (fibrosis and cirrhosis) which are characterized by dramatic increases in collagen deposition [199]. Given the dominance of collagenous changes in the hepatic ECM during fibrosis/cirrhosis, many studies and previous reviews have focused on the underlying changes in collagen deposition.

Transitional Extracellular Matrix Changes and Inflammatory Liver Damage

Hepatic ECM changes in response to alcohol are not limited to fibrogenesis per se. Indeed, it is becoming increasingly understood that the hepatic ECM responds dynamically to stress (Fig. 3.5). For example, changes in the expression of ECM proteins such as complement and fibrin (see below) and fibronectin [200, 201] have been observed in models of hepatic inflammation. Importantly, blocking these ECM changes blunts, at least in part, hepatic injury in these models. Other alterations of the ECM include changes in the deposition and distribution of laminin and vitronectin [202]. These dynamic changes in the matrix likely play key roles in mediating and coordinating the (patho)physiologic responses of the tissue.

PAI-1 and Hepatic Fibrosis

Plasmin degrades other extracellular matrix (ECM) proteins, such as laminin, proteoglycan, and type IV collagen [203–205] in addition to fibrin. Furthermore, plasmin activates MMPs, thereby indirectly degrading other ECM proteins [206]. Thus, by blocking plasminogen activation, PAI-1 could significantly alter the ECM. Indeed, a protective effect of pharmacologic/genetic prevention of PAI-1 induction has been observed in models of renal, pulmonary, and vascular remodeling, which share mechanisms with hepatic fibrosis [207–209]. The effect of knocking PAI-1 on hepatic fibrosis was first determined in a bile duct ligation model [210, 211]. In brief, PAI-$1^{-/-}$ mice were dramatically protected against ECM accumulation and fibrosis. A similar profibrotic effect of PAI-1 was observed in the AngII model of hepatic fibrosis [212], in which the protective effects of knocking out PAI-1 correlated with elevated degradation of ECM.

In contrast, liver damage and fibrosis were dramatically enhanced in PAI-1 knockout mice after chronic CCl_4 exposure [213]. This may be secondary to an impaired hepatocyte proliferative response. A proproliferative role of PAI-1 was observed in mouse liver in experimental acetaminophen toxicity [214]. It is hypothesized that this impaired ability of the PAI-1 knockout liver to restitute itself enhances the amount of liver damage and accelerates the fibrotic process during chronic CCl_4 exposure. The exact contribution of PAI-1 to human ALD awaits clinical trials of inhibitors of this protein, correlations of PAI-1 activity with liver injury (for instance, in alcoholic hepatitis), or implication of the PAI-1 locus in genome-wide association studies that are now under way.

The Complement Cascade and ALD

The complement system is a complex component of the innate immune system. It consists of soluble and membrane-bound proteins functioning by stepwise proteolytic activation. The complement cascade can be activated via three pathways: the classical, lectin, or alternative pathways. Activation of any of these pathways can lead to the cleavage of C3 and subsequent downstream activation of C5 and the rest of the terminal pathway. Hence, C3 plays a pivotal role in the complement system. In addition to producing complement proteins, cells in the liver also express complement factor receptors, as well as intrinsic regulatory proteins. Under basal

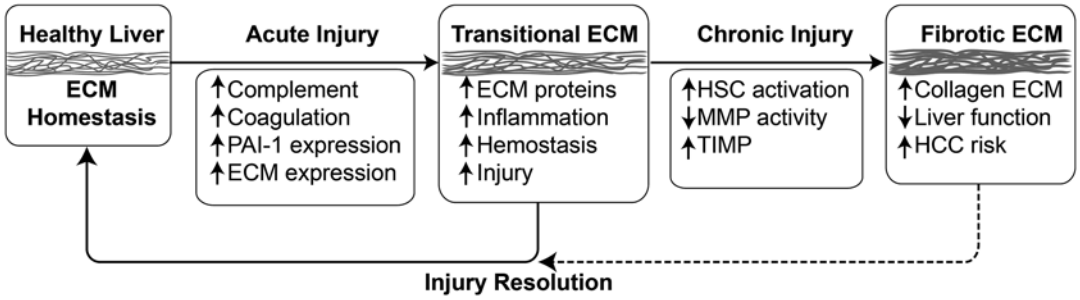

Fig. 3.5 Matrix alterations in response to acute and chronic damage in the liver. Although remodeling in response to chronic injury (i.e., fibrosis) is well known in the context of chronic liver injury, the hepatic extracellular matrix (ECM) also responses dynamically to acute stress. Key events in response to acute stress include activation of the complement and coagulation cascades, as well as induction of key genes (e.g., PAI-1 and osteopontin) that directly or indirectly mediate qualitative and quantitative changes to the ECM. These acute responses can be viewed as an arm of the wound-healing response and facilitate recovery from damage, which resolves once the damage is repaired. However, under conditions of chronic injury, these changes contribute to activation of a significant remodeling response that leads to scar formation. This latter response is known as fibrogenesis in the liver. Other abbreviations: *MMP* matrix metalloproteases, *TIMP* tissue inhibitors of metalloproteases, *PAI-1* plasminogen activator inhibitor-1, *HCC* hepatocellular carcinoma

conditions, Kupffer cells and HSCs express C3a and C5a receptors [215]. C5a receptor expression can be induced in proliferating hepatocytes or in response to inflammatory cytokines [215].

Recently, it has been suggested that complement activation contributes to the pathogenesis of various forms of liver diseases, including ALD [215]. In animal models, it was shown that chronic alcohol feeding increased activation of C3 [216], as well as increased accumulation of C3 or its proteolytic end product C3b/iC3b/C3c in the liver. Mice deficient in C3 and C5 are protected against alcohol-induced steatosis and increased transaminases [216]. In line with these findings, chronic alcoholic liver injury is exacerbated in mice lacking CD55/DAF, a complement regulatory protein that prevents the assembly of C3 convertase complexes and blocks the formation of the final membrane attack complex [216]. Moreover, alcohol feeding activates the classical complement pathway via C1q binding to apoptotic cells in the liver. Deletion of C1q prevents ethanol-induced complement activation, blunts TNFα and IL-6 expression in the liver, and reduces alcohol-induced liver damage [217]. In humans, the level of complement is increased in alcoholics during withdrawal, but is reduced in alcoholics with cirrhosis [218]. Therefore, future studies of complement in human ALD may help identify new therapeutic targets to treat this disease.

The Coagulation Cascade and Fibrin ECM in ALD

The liver is the major organ regulating the fibrin coagulation system. Fibrin metabolism is regulated via two pathways, coagulation and fibrinolysis [219]. Inhibition of fibrinolysis by plasminogen activator inhibitor-1 (PAI-1) can cause fibrin ECM to accumulate, even in the absence of enhanced fibrin deposition by the thrombin cascade [220]. Hepatic injury often involves dysregulation of the coagulation cascade and fibrinolysis, resulting in the formation of fibrin clots in the hepatic sinusoids [221, 222]. Fibrin clots block the blood flow within the hepatic parenchyma, causing microregional hypoxia and subsequent hepatocellular death [223, 224].

Fibrin ECM not only serves as a physical structure but also binds/interacts with several cellular biomolecules. One family of receptors that mediate these interactions is the integrins.

Integrins transfer information from the ECM to the cell, allowing rapid and flexible responses to changes in the environment. Integrins play a myriad of roles within the body, including proliferation/angiogenesis, maintenance of differentiation, as well as inflammation and apoptosis [225, 226]. Integrins are found on almost all cell types in the liver. Therefore, altering the composition of the ECM has the potential to alter inflammatory signaling in the liver via a myriad of mechanisms.

Elevated PAI-1 levels and hypofibrinolysis are common during the development of ALD [227]. Indeed, PAI-1 levels during disease development are a predictor of later severity [228]. A recent human study further supports the hypothesis that PAI-1 plays a critical role in ALD [229]. However, few clinical studies have focused on the potential of inhibiting PAI-1 to slow the development of ALD. Indeed, it is well known that bleeding as well as thrombosis is common in end-stage liver disease [230], and there has been the slow realization that cirrhosis is a hypercoagulable state. In light of this, it is intriguing that anticoagulation with enoxaparin was reported to prevent decompensation in cirrhotics with portal vein thrombosis [231].

Osteopontin

Osteopontin, also designated secreted phosphoprotein-1 (SPP-1), has been intensively studied for many years. It predominantly serves as an extracellular structural glycoprotein. Its synthesis is greatly upregulated in animal and human ALD by the increased exposure to LPS and has been linked to the activation of HSC and liver fibrosis (reviewed by Seth et al. [232, 233]) and with poor outcomes in alcoholic hepatitis [234]. However, very recent work with genetically modified mice has indicated that overexpression of SPP-1 prevents early ALD, in part by way of its ability to bind LPS [235], and knockout of SPP-1 promotes the neutrophilic infiltration of the liver in a model of alcoholic hepatitis [236]. The roles of osteopontin at various stages of ALD are thus open to further investigation, which will obviously bear on any therapeutic application of this protein.

Summary

Clearly, heavy alcohol use affects a myriad of signaling pathways and deranges metabolism (in particular the handling of lipids), sensitizes inflammatory cells to LPS, stimulates release of a number of cytokines and other products of innate immunity, promotes apoptosis and necroptosis of hepatocytes, and, in common with numerous other causes of hepatic injury, stimulates the deposition of extracellular matrix, leading in the most severe cases to either death from hepatic failure (in the case of acute alcoholic hepatitis) or the development of fibrosis and cirrhosis. It is no surprise that therapies addressing only one aspect of alcoholic liver injury do not uniformly improve the outcomes of the most severely affected patients, and this growing body of work on the mechanisms of alcoholic liver injury suggests that treatment interrupting several of these pathways or augmenting protective responses, although difficult in practice to study, may offer the best hope for the care of patients who abuse alcohol.

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Pathogenesis of NAFLD and NASH

4

Jacquelyn J. Maher

Abbreviations

ASC	Apoptosis-associated speck-like protein containing a caspase activation and recruitment domain
ASMase	Acidic sphingomyelinase
ATF6	Activating transcription factor 6
BMAL	Brain and muscle aryl-hydrocarbon receptor nuclear translocator-like
CB2	Cannabinoid receptor 2
CCL2	C-C chemokine ligand 2
CHOP	C-EBP homologous protein
ChREBP	Carbohydrate response element-binding protein
CLOCK	Circadian locomotor output cycles kaput
CRY	Cryptochrome
c-Src	Proto-oncogene tyrosine-protein kinase
DAG	Diacylglycerol
DAMP	Damage-associated molecular pattern
DEN	Diethylnitrosamine
DNL	De novo lipogenesis
EGFR	EGF receptor
ER	Endoplasmic reticulum
Fas	Fas/CD95 receptor
FasL	Fas ligand
FIAF	Fasting-induced adipocyte factor
FATP5	Fatty acid transporter 5
FOXO1	Forkhead box O1
FXR	Farnesoid X receptor
HGF	Hepatocyte growth factor
IL-1β	Interleukin-1β
IRE1	Inositol-requiring enzyme 1
JNK	Jun N-terminal kinase
LPAAT	Lysophosphatidic acid acyltransferase
MLKL	Mixed lineage kinase domain-like protein
MRC	Mitochondrial respiratory chain
mTOR	Mammalian target of rapamycin
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NCAN	Neurocan
NLRP	NOD-like receptor proteins
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular pattern
PER	Period
PERK	RNA-dependent protein kinase-like ER kinase
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

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PGC1β	Peroxisome proliferator-activated receptor gamma coactivator 1-beta
PKC	Protein kinase C
PNPLA3	Patatin-like phospholipase domain-containing 3
PPARδ	Peroxisome proliferator-activated receptor-δ
PRR	Pattern recognition receptor
RANTES	Regulated on activation, normal T cell expressed and secreted (CCL5)
RIP	Receptor-interacting protein
RORα	RAR-related orphan receptor A
ROS	Reactive oxygen species
SCAP	SREBP cleavage-activating protein
SHP	Short heterodimer partner
SIBO	Small intestinal bacterial overgrowth
SREBP1	Sterol regulatory element-binding protein-1
TAK1	Transforming growth factor β-activated kinase 1
TCA	Tricarboxylic acid cycle
TLR	Toll-like receptor
TM6SF2	Transmembrane 6 superfamily member 2
TNFR1	TNF receptor-1
TRAIL	TNF-related apoptosis-inducing ligand
XBP1	X-box protein-1

Pathogenesis of Hepatic Steatosis

The liver plays an important role in whole-body energy homeostasis. Among the liver's contributions to metabolism is its ability to synthesize and package excess fuel as neutral lipid. Under normal circumstances, surplus lipids are exported out of the liver to adipose tissue for short- or long-term storage; however, under pathologic conditions this process is deranged, causing the liver to become an ectopic reservoir for fat. Hepatic fat accumulation, or steatosis, is the defining feature of NAFLD and an essential element of the disease NASH. This section summarizes the pathologic events that stimulate and perpetuate hepatic steatosis and reviews how certain genetic and environmental factors influence this process.

Adipose Tissue Dysfunction as a Trigger to Hepatic Steatosis

Hepatic steatosis almost always occurs in the setting of obesity. The progressive expansion of fat depots in overweight individuals causes adipose tissue dysfunction and inflammation, which compromises the ability of adipocytes to perform their normal lipid storage function (Fig. 4.1) [1, 2].

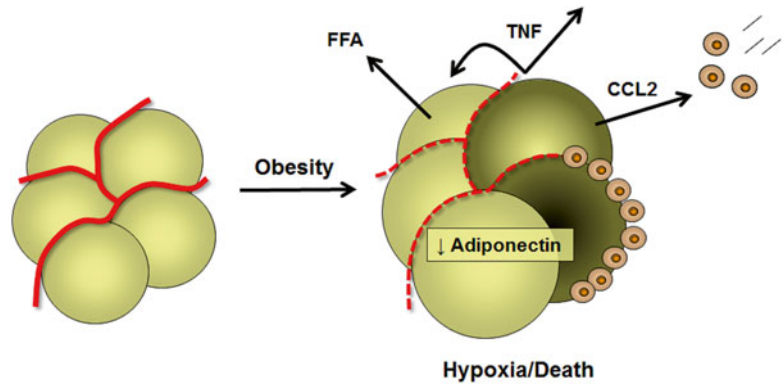

Fig. 4.1 Adipocyte dysfunction in obesity. Adipocytes hypertrophy and enlarge during obesity. This can lead to an imbalance between blood flow and oxygen demand, resulting in hypoxia and cell death. Dysfunctional adipocytes produce pro-inflammatory cytokines including TNF and CCL2. TNF induces insulin resistance, which leads to lipid hydrolysis and release of fatty acids into the circulation. CCL2 recruits macrophages, which produce more cytokines and amplify the inflammatory and insulin-resistant state

Adipocyte dysfunction in obesity may be triggered in part by hypoxia as proliferating and enlarging adipocytes outstrip their blood supply [3]; these hypertrophied and hypoxic adipocytes are highly sensitized to cell death [4–6]. Compromised adipocytes produce pro-inflammatory cytokines such as TNF [7, 8]. TNF impairs adipocyte insulin signaling, leading to triglyceride lipolysis and release of free fatty acids into the circulation. In addition, dysfunctional and dying adipocytes secrete chemokines such as C-C chemokine ligand 2 (CCL2) that recruit macrophages to the adipose tissue [9, 10]. The infiltrating macrophages amplify the inflammatory milieu by producing more cytokines and perpetuating the already established insulin-resistant state. As adipose tissue inflammation progresses, adipocytes decline in their production of adiponectin, an adipokine that stimulates lipid storage in adipocytes and promotes insulin sensitivity [11–14]. Under conditions of adipose tissue dysfunction, the liver is exposed to free fatty acids arising from adipocyte lipolysis as well as cytokines that leak into the circulation from adipose tissue. Together these can promote hepatic steatosis [15, 16].

Epidemiologic studies suggest that certain adipose tissue depots have a greater potential for dysfunction in obesity than others. Specifically, visceral fat is highly prone to insulin resistance [17], and visceral obesity often correlates with hepatic steatosis or liver injury [18–22]. Interestingly, though, the connection between visceral obesity and hepatic steatosis in humans may not be a direct consequence of insulin resistance. This was highlighted in the Dallas Heart Study, in which African-Americans with visceral obesity exhibited more insulin resistance than Hispanics but had less hepatic steatosis [21].

Although hepatic steatosis typically follows obesity, one important exception to this rule is that hepatic steatosis and even steatohepatitis also occur in patients with lipodystrophy, characterized by a paucity of adipose tissue [23–25]. This paradox can be explained by the fact that normal adipose tissue produces leptin, adiponectin, and other adipokines whose functions are to promote lipid homeostasis [23, 26]. In the setting of lipodystrophy, the absence of functional adipose tissue induces the same adverse consequences for the liver as does the presence of dysfunctional adipose tissue in obesity. When viewed together, both diseases underscore that some amount of healthy adipose tissue is required to maintain metabolic homeostasis in the liver [27].

Adipose Tissue Insulin Resistance and Downstream Consequences for the Liver

As mentioned above, insulin-resistant adipose tissue loses its ability to store lipid and releases free fatty acids into the circulation. In an effort to compensate for adipose tissue insulin resistance, the pancreas produces more insulin, which leads to hyperinsulinemia. Thus, in obesity, the liver encounters excesses of both free fatty acids and insulin (Fig. 4.2a). Liver cells take up fatty acids through the transport proteins FATP5 (fatty acid transport protein 5) and CD36 [28, 29]. Both proteins are upregulated in obesity [30, 29], priming the liver to accommodate the enhanced flux from adipose tissue. Once inside the liver, fatty acids are steered toward storage in the form of triglyceride, which provides an immediate and direct route to hepatic steatosis. During the process of triglyceride synthesis, the levels of several intermediates in the triglyceride pathway also rise. Of particular importance are diacylglycerols (DAG), which can activate protein kinase C epsilon (PKCε). PKCε, in turn, can interfere with the normal function of insulin receptors on hepatocytes [31, 32], resulting in a state of hepatic insulin resistance.

In the liver, insulin is an important regulator of two metabolic functions: gluconeogenesis and lipid synthesis. Gluconeogenesis is normally suppressed by insulin, and thus, hepatic insulin resistance is marked by an increase in hepatic glucose production (Fig. 4.2b). Hepatic lipid synthesis is normally enhanced by insulin; thus, hepatic lipid synthesis should be decreased in an insulin-resistant liver. Curiously, this is not the case, and the paradox has been referred to as “selective insulin

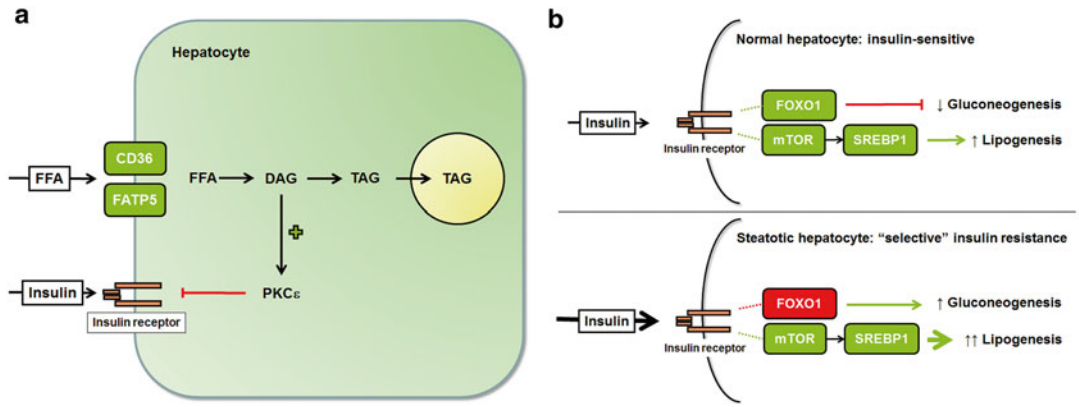

Fig. 4.2 Hepatic steatosis promotes hepatic insulin resistance. (**a**) Insulin resistance outside the liver exposes the liver to high circulating levels of free fatty acids (FFA) and insulin. FFA are taken up by hepatocytes via the transporters CD36 and FATP5. Once inside the cell, the FFA are incorporated into diacylglycerols (DAG) and triacylglycerols (TAG). Excess DAG within hepatocytes activates PKCε, which impairs insulin signaling. (**b**) In normal hepatocytes, insulin stimulates lipogenesis and suppresses gluconeogenesis. Lipogenesis is upregulated by mTOR and SREBP1; gluconeogenesis is suppressed by FOXO1. In obesity, circulating insulin levels are high, and hepatocyte insulin signaling is impaired, at least partially. Insulin-mediated FOXO1 activation is impaired, resulting in uncontrolled gluconeogenesis; insulin-mediated activation of mTOR and SREBP1, however, is not suppressed, which leads to enhanced lipogenesis. These discordant responses have been termed "selective insulin resistance"

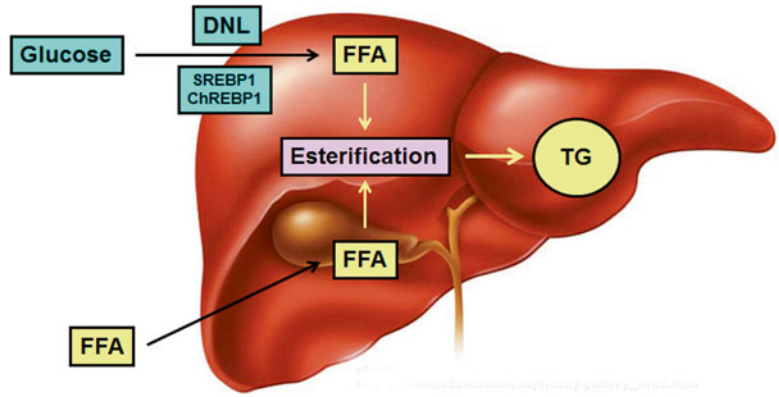

Fig. 4.3 Hepatic steatosis arises from de novo lipogenesis as well as fatty acid uptake. Excess free fatty acids (FFA) in the liver are esterified to triglyceride (TG) for storage. Some FFA are taken up by the liver from the circulation; others are produced in the liver from glucose, through de novo lipogenesis (DNL). DNL is stimulated by insulin (via SREBP1) and glucose (via ChREBP1)

resistance" (Fig. 4.2b) [33]. The fact that hepatic lipid synthesis remains sensitive to insulin in a state of hepatic lipid excess whereas gluconeogenesis does not implies that the two metabolic pathways must diverge at some point. Experimental studies indicate that gluconeogenesis is controlled by forkhead box O1 (FOXO1) downstream of the insulin receptor, whereas lipogenesis is controlled by mammalian target of rapamycin (mTOR) [34]. This leaves open the possibility that in a state of hepatic insulin resistance, other factors besides insulin could continue to stimulate hepatic lipogenesis by activating mTOR.

The lipogenic program activated in the liver during obesity involves more than the esterification of adipocyte-derived fatty acids to triglyceride. It also involves the synthesis of new fatty acids from carbohydrate (de novo lipogenesis; DNL) (Fig. 4.3). The enzymes responsible for DNL are regulated in part by insulin, through the action of the transcription factor sterol regulatory element-binding protein-1 (SREBP1) [35]. Importantly, SREBP1 is active in the liver even during times of hepatic insulin resistance (see above, "selective insulin resistance"). DNL enzymes are also regulated by glucose through

the action of a second transcription factor, carbohydrate response element-binding protein (ChREBP). Together, SREBP1 and ChREBP promote hepatic DNL in response to high-carbohydrate feeding, hyperglycemia, and hyperinsulinemia [35]. In the normal liver, only about 4 % of hepatic triglyceride derives from DNL. In obese individuals, this proportion increases as fatty liver progresses, to as much as 25 % [36, 37]. Because the flux of fatty acids to the liver from adipose tissue does not decline as DNL rises [38, 39], adipose tissue-derived fatty acids must over time be diverted to pathways other than triglyceride synthesis. Evidence indicates they are shuttled to oxidation, to provide the energy necessary to drive the processes of gluconeogenesis and DNL [39].

ER Stress and Its Contribution to Hepatic Steatosis

Once lipid accumulation begins in hepatocytes, it can set into motion events that prompt even more steatosis. A key contributor to this process is the endoplasmic reticulum (ER), an organelle that plays a central role in cellular metabolism. The classical function of the ER is to execute post-translational processing of proteins. The ER can become stressed, either from an increased demand for protein folding or from derangements in the internal environment of the organelle [40]. Stress in the ER prompts adaptive responses involving the activation of one or more ER membrane proteins as shown in Fig. 4.4. Although these responses are designed to

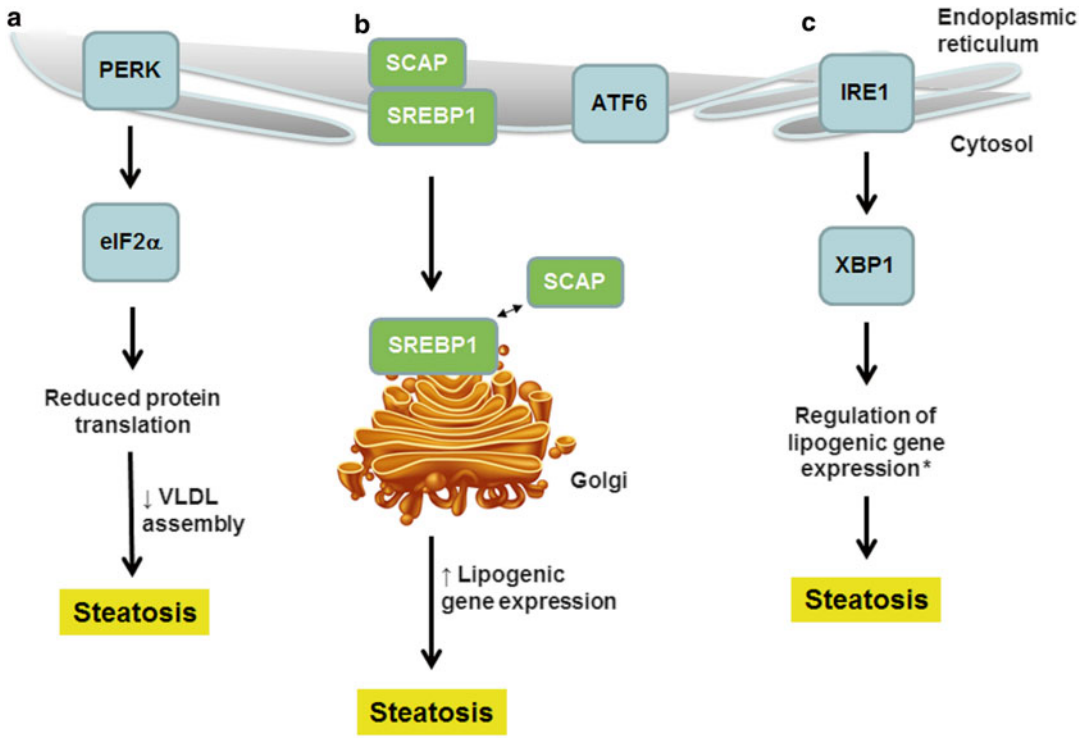

Fig. 4.4 Endoplasmic reticulum stress contributes to hepatic steatosis. ER stress transducers, positioned within the ER membrane, respond to excess fatty acids. (**a**) Activation of PERK leads to a reduction in protein synthesis, including the synthesis of apolipoproteins necessary for VLDL assembly. (**b**) SREBP1, although not itself an ER stress transducer, is activated by ER stress similarly to ATF6. ER stress promotes migration of SREBP1 to the Golgi where it becomes activated by dissociation from SCAP; SREBP1 stimulates lipogenic gene expression. (**c**) Activation of IRE1 leads to activation of XBP1, a transcription factor that regulates lipogenic gene expression. These three events promote hepatic steatosis (*whether XBP1 stimulates or suppresses lipogenic gene expression is currently debated)

maintain ER homeostasis, they may have undesirable side effects. In hepatocytes, for example, exposure to excess fatty acids places stress on the ER by creating demand for the synthesis of apolipoproteins needed for lipid export as VLDL [18]. This activates the RNA-dependent protein kinase-like ER kinase (PERK), which signals downstream events that result in a suppression of cellular protein synthesis. Although well intentioned, this response prevents rather than facilitates fatty acid export; the ensuing outcome is fatty acid retention and steatosis.

Another important consequence of ER stress is the transformation of SREBP1 from an inactive to a transcriptionally active form [41]. SREBP1, like the ER stress proteins shown in Fig. 4.4, normally resides in the ER membrane; there it is maintained in an inactive state through interactions with a binding partner named SCAP (SREBP cleavage-activating protein) [42]. Under conditions of ER stress, the SREBP1–SCAP complex moves from the ER to the Golgi apparatus; the complex then dissociates, and SREBP1 is released from the Golgi membrane by proteolytic cleavage, enabling its translocation to the nucleus [43]. A connection between ER stress and SREBP1 activation was first theorized when it was noted that the same steps of Golgi transport and membrane release required to process SREBP1 are also needed to activate the ER stress transducer ATF6 (activating transcription factor 6) [44]. Subsequently, experiments showed that ER stress can activate SREBP1 independently of its classic stimulus, insulin. This was a key finding because it revealed yet another potential mechanism by which SREBP1 and hepatic lipogenesis could persist in fatty livers in the face of hepatic insulin resistance. Importantly, if hepatic steatosis induces ER stress in the liver, and ER stress in turn activates SREBP1, this creates a positive feedback loop for the persistence and progression of fat accumulation in the livers of obese individuals once the process is initiated.

Among the adaptive responses to ER stress is an increase in the synthesis of cellular lipids. Lipid synthesis enables a stressed ER to expand its volume and processing capacity [45]. Stress-related lipid synthesis is mediated primarily through inositol-requiring enzyme 1 (IRE1) and its downstream target X-box protein-1 (XBP1) [46–48]. These two molecules have the potential to influence several aspects of lipid metabolism, including fatty acid and triglyceride synthesis, lipid hydrolysis, and lipid transport [49, 50]. In the liver, the lipid alterations that ensue from activation of IRE1 and XBP1 may not always be adaptive. Instead, they may contribute to hepatic steatosis. This was first discovered when disruption of the pathway by silencing XBP1 in the livers of obese mice improved hepatic steatosis [51]. Recent research into the role of the IRE1–XBP1 axis in hepatic lipid homeostasis has cast some doubt on the ability of IRE1 and XBP1 to stimulate lipogenesis directly. In fact, one group suggests that IRE1–XBP1 signaling is actually necessary for the successful lipidation of VLDL particles [49]. In this case, activation of IRE1–XBP1 would prevent, rather than promote, hepatic steatosis.

Although at present there remains some uncertainty about the importance of ER stress in comparison to other mechanisms of obesity-related hepatic steatosis (Table 4.1), there is ample experimental evidence that ER stress regulates hepatic lipid homeostasis at multiple levels

Table 4.1 Factors leading to hepatic steatosis in obesity

Factor	Mechanism
Increased flux of FFA from the adipose tissue to liver	Insulin resistance in adipose tissue promotes lipolysis and FFA release
Increased hepatic uptake of FFA from the circulation	Upregulation of hepatic fatty acid transporters CD36 and FATP5
Insulin-mediated stimulation of hepatic lipogenesis	Insulin-dependent stimulation of the lipogenic transcription factor SREBP1
Glucose-mediated stimulation of hepatic lipogenesis	Glucose-dependent stimulation of the lipogenic transcription factor ChREBP1
ER stress-mediated stimulation of hepatic lipogenesis	Insulin-independent activation of SREBP1; IRE1–XBP1 activation leading to induction of lipogenic genes
ER stress-mediated inhibition of hepatic lipid export	PERK-mediated reduction of ApoB synthesis, resulting in reduced VLDL synthesis

through multiple signal transducers. ER stress signaling can also lead to hepatocyte death in a fatty liver; this will be discussed below.

Autophagy and Steatosis

Autophagy is a process that degrades dysfunctional cellular constituents and maintains cellular energy stores. During autophagy, material is captured in a membrane-bound vesicle called an autophagosome, which then fuses with a lysosome permitting digestion of the contents and releases back into the cytosol. Recently, it was discovered that autophagy plays an important role in lipid homeostasis in adipocytes [52] and hepatocytes [53]. Autophagy is considered particularly important to hepatocytes as a means of accessing the energy stored in cellular lipid droplets, because these cells are not equipped with high concentrations of lipases [54]. Fatty acids mobilized from lipid droplets via autophagy are routed to mitochondria for oxidation. The importance of autophagy to normal hepatic lipid metabolism was documented in experiments demonstrating that inhibition of autophagy leads to spontaneous hepatic steatosis [53]. More importantly, and directly pertinent to fatty liver disease, it was discovered that loading normal liver cells with lipid impairs autophagy [53]. This prompted further investigation to determine whether autophagy is dysregulated in a fatty liver in vivo, and the suspicion was confirmed [53]. At present, it does not appear that abnormal autophagy is a primary cause of hepatic steatosis, but impairments in autophagy that result from steatosis can enhance or perpetuate hepatic lipid accumulation. Dysregulated autophagy can also promote cell death in a fatty liver; this is discussed later (see section "Mechanisms of Cell Death in NASH").

Regulation of Steatosis by Bile Acids

Bile acids exert important influences on lipid metabolism in the liver [55]. The principal means by which bile acids control lipid homeostasis is through interactions with the farnesoid X receptor (FXR) (Fig. 4.5). Bile acid-induced stimulation of FXR leads to activation of the downstream target, short heterodimer partner (SHP); SHP, in turn, suppresses SREBP1, which results in inhibition of hepatic lipogenesis. FXR also activates

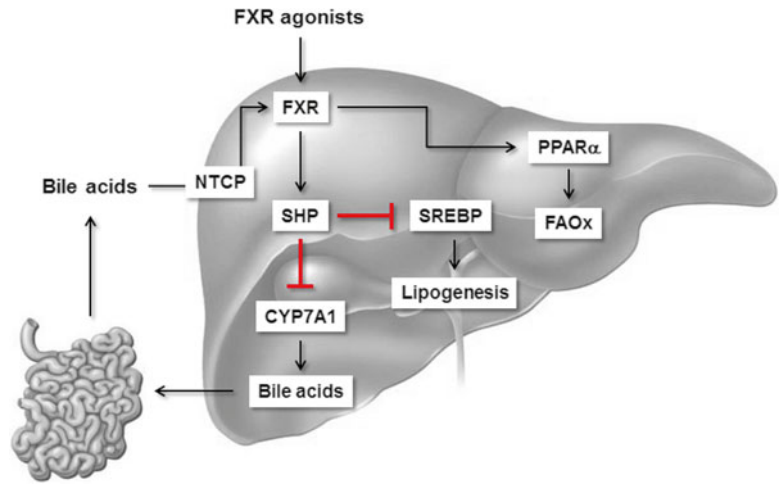

Fig. 4.5 Regulation of hepatic lipid metabolism by bile acids. Bile acids entering the liver via the NTCP transporter stimulate the nuclear hormone receptor, FXR. FXR then activates SHP, which suppresses bile acid synthesis and also suppresses hepatic lipogenesis by reducing SREBP1 activity. FXR also activates PPARα, which stimulates fatty acid oxidation (FAOx). Taken together, FXR activation suppresses hepatic lipid synthesis and enhances fatty acid oxidation. Pharmacologic FXR agonists have similar beneficial effects on hepatic lipid metabolism

PPARα, a master inducer of fatty acid oxidation. This means that bile acid signaling should limit hepatic steatosis by two complementary means. The tonic influence of bile acids on hepatic lipid homeostasis has been demonstrated in mouse studies, which reveal that knockout of FXR leads to hepatic steatosis and steatohepatitis [56]. On the other hand, there is only limited evidence that an alteration in FXR is a root cause of fatty liver disease in humans. One study reports that patients with hepatic steatosis and NASH have low levels of FXR in the liver [57]. Another looked for modulation of hepatic lipid levels as a consequence of a polymorphism in the gene encoding FXR, but found none [58]. Recently, however, it was discovered that high-fat feeding in mice induces a gene named yin yang 1 (YY1) that suppresses FXR [59]. YY1 is upregulated by TNF [60], and thus, the pro-inflammatory state of obesity may be responsible for YY1 induction and FXR suppression.

Even if fatty liver disease is not attributable to a defect in FXR signaling, FXR agonism improves hepatic steatosis [61–64]. The therapeutic potential of synthetic bile acids for fatty liver disease is discussed in Chap. 9B.

Influence of Genetics on Hepatic Steatosis

There is a heritable element to NAFLD and NASH, based on evidence that the disease clusters in families, is concordant among monozygotic twins, and exhibits clear ethnic variations in disease risk (reviewed in [65]). The genetics of NAFLD and NASH are discussed in detail in this chapter. Because the current chapter focuses on the pathogenesis of NAFLD and NASH, it is worth highlighting a few examples of NASH-related gene polymorphisms here, focusing on the mechanism by which they might contribute to disease pathogenesis. The most notable genetic alteration that has been linked to NASH is a C → G polymorphism leading to an I148M substitution in the gene PNPLA3 (patatin-like phospholipase domain-containing 3) [66–68]. PNPLA3 encodes adiponutrin, a protein located in the membrane fraction of hepatocytes and adipocytes. Investigators have gone to great lengths to elucidate how mutant adiponutrin promotes steatosis. In one study, mice were engineered to overexpress human I148M adiponutrin. They exhibited enhanced hepatic lipogenesis, which led to the conclusion that adiponutrin functions primarily as lysophosphatidic acid acyltransferase (LPAAT) that generates lysophosphatidic acid in the pathway leading to triacylglycerols [69]. However, in a more recent study, the I148M mutation was "knocked into" the native mouse gene. Using this approach, the mice displayed impaired triglyceride lipolysis, prompting the opposite conclusion that adiponutrin is primarily a triglyceride lipase [70]. Although more research is required to clarify the precise role of mutant adiponutrin in fatty liver disease, some observations are worth noting. First, the I148M knock-in mutation does not cause spontaneous hepatic steatosis in mice, but does enhance steatosis in response to high-carbohydrate feeding [70]. This is consistent with the classification of PNPLA3 as a modifier of NASH in humans [71]. Second, although overexpression or knock-in of mutant adiponutrin induces hepatic steatosis in mice, as yet there is no report of its ability to induce experimental steatohepatitis.

A gene named transmembrane 6 superfamily member 2 (TM6SF2) was recently discovered in an exome-wide association study to be associated with hepatic steatosis [72]. The link between TM6SF2 and NAFLD is believed to supersede that of a nearby gene, neurocan (NCAN), which was uncovered in an earlier GWAS study [67]. Hepatic steatosis is associated with an A → G polymorphism in TM6SF2 leading to a glutamate-to-lysine substitution at amino acid 167. The TM6SF2 polymorphism has already been confirmed by other groups as relevant not only to steatosis [73] but also NASH and cirrhosis [74]. TM6SF2 is a transmembrane protein of unknown function, but in vitro studies indicate the E167K variant has reduced stability. Knockdown of TM6SF2 in liver cell lines and mouse liver in vivo results in steatosis, particularly following a carbohydrate challenge. Importantly, TM6SF2-

deficient mice have reduced levels of circulating lipids, which suggests the protein facilitates hepatic VLDL secretion [72]. These examples illustrate how individual gene polymorphisms, each with a strong association to NAFLD but an independent effect on the liver, can impact the NAFLD phenotype. They also underscore how multiple independent modifiers in an individual subject might complicate disease severity and response to treatment.

Influence of the Gut Microbiome on Hepatic Steatosis

It is now evident that microbial communities in the intestine are important determinants of metabolism. Pertinent to obesity are observations that diet affects the gut microbiome and the gut microbiome in turn affects nutrient absorption. Specifically, overeating stimulates the intestinal overgrowth of bacteria belonging to the phylum Firmicutes [75, 76], which are efficient at extracting nutrients from the diet [77]. Some of the adverse effects of round-the-clock eating may also stem from alterations in the gut microbiome [78]. Thus, microbial derangements induced by alterations in eating behavior create a situation that enhances the potential for obesity and its complications.

In addition to having an altered colonic microbiome, overweight individuals with fatty livers often display small intestinal bacterial overgrowth (SIBO) [79, 80]. SIBO is thought to arise from impaired intestinal motility in obesity, which can result from a reduction in the hormone ghrelin [81]. Toxins or inflammatory mediators elaborated by these abundant organisms can compromise intestinal barrier function by degrading tight junction proteins [80, 82]. This allows potentially harmful compounds from the intestine to invade the portal circulation and reach the liver, where they can stimulate NAFLD and NASH.

Two intestinal compounds that have been implicated in the development of NAFLD and NASH are bacterial endotoxin and ethanol. Endotoxin, which can enter the portal circulation through a compromised intestinal barrier, is readily detectable in the blood of patients with hepatic steatosis and NASH [80, 83]. Importantly, endotoxin has even been identified in the blood of healthy persons fed a high-energy diet for a brief period [82, 84]. This suggests that alterations in the intestine can occur very early in the evolution of obesity and NAFLD. Ethanol, a by-product of bacterial fermentation in the gut, can be absorbed through an intact intestinal epithelium. Like endotoxin, ethanol is detectable in the circulation of patients with NAFLD [85, 86] and has the potential to promote hepatic steatosis or steatohepatitis (see Chap. 9A on Alcoholic Liver Disease).

Intestinal bacteria can also perform other functions that lead to undesirable effects on hepatic lipid homeostasis. One is the ability to convert choline into methylamines. This property, which is characteristic of microbes that overgrow in obesity [87], can lead to choline depletion in the intestine and choline deficiency in the host. Choline deficiency is well known to promote hepatic steatosis [88]; it does so by limiting the synthesis of phosphatidylcholine, critical to the assembly of VLDL particles [89]. Intestinal bacteria also suppress the synthesis of fasting-induced adipocyte factor (FIAF), an inhibitor of lipoprotein lipase [90, 91]. When FIAF is reduced, adipose tissue lipolysis is increased, which results in an increase in fatty acid delivery to the liver.

Lastly, gut microbes can influence metabolism through effects on bile acids. Gut bacteria can modify bile acid structure and activity; conversely, bile acids can influence microbial homeostasis in the gut. The interplay between microbes and bile acids, as well as other topics relevant to the gut microbiome in fatty liver disease, is reviewed in [92–94].

Behavioral Factors Affecting Hepatic Steatosis

Intake of Specific Dietary Nutrients Although there is an undeniable relationship between caloric intake and obesity/fatty liver disease,

studies suggest not all calories are alike. Indeed, certain nutrients have a specific propensity to promote steatosis and steatohepatitis. Among the dietary sugars, fructose is considered particularly noxious because it induces visceral obesity and insulin resistance more readily than glucose [95]. This is likely attributable to the unregulated nature of fructose entry into DNL, as well as the ability of fructose to directly activate SREBP1 independently of insulin [96, 97]. Epidemiologic studies implicate fructose intake as a risk factor for NAFLD in adults and children [98–100]. Moreover, adding fructose to a standard diet in the form of sweetened beverages can provoke hepatic steatosis in as few as 7 days [101]. Although there remains some controversy whether fructose is more harmful to the liver than other sugars [102], head-to-head comparisons in animal models support this theory [103].

Of the dietary fats, trans fats, which are man-made unsaturated fats, have a specific tendency to provoke NAFLD. Data supporting the connection between trans fats and NAFLD come largely from animal studies. In mice, trans fats produce more steatosis and liver injury than saturated or standard polyunsaturated fats when consumed in isocaloric amounts [104, 105]. Interestingly saturated fats, which are singled out as important risk factors for cardiovascular disease [106], do not consistently induce more hepatic steatosis than unsaturated fats in mice when incorporated into experimental diets [107, 108]. When saturated fats are paired with simple sugars, though, the two nutrients synergize to promote hepatic steatosis (Maher JJ et al., unpublished). This may be due to the ability of saturated fat to stimulate hepatic DNL by activating SREBP1 through peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1α) [109]. In humans, saturated fat-rich food supplements promote more hepatic steatosis than polyunsaturated fat-rich supplements [110]. Overall, the available information to date indicates that saturated fats and trans fats both have greater potential than other types of fat to induce hepatic steatosis and liver injury.

Cholesterol consumption, which has been linked to the development of chronic liver disease in humans [111], contributes directly to the pathogenesis of experimental NASH. Mice fed diets containing 0.2–2.0 % cholesterol by weight consistently develop more severe steatohepatitis than those fed diets lacking cholesterol [112–116]. Fatty liver disease in cholesterol-fed mice coincides with the accumulation of free cholesterol in the liver [115], which may contribute directly to liver cell injury (see section "Mechanisms of Cell Death"). A similar rise in hepatic free cholesterol has been reported in humans with NAFLD and NASH [117]. The fact that cholesterol is elevated in NAFLD as well as NASH could indicate this derangement is a prerequisite to disease progression.

The Diurnal Clock and the Timing of Food Intake Many bodily functions are subject to diurnal control through the action of the so-called clock genes. A 24-h oscillatory cycle of gene expression is maintained by the transcriptional activators CLOCK (circadian locomotor output cycles kaput) and BMAL (brain and muscle aryl-hydrocarbon receptor nuclear translocator-like) and the transcriptional repressors PER (period) and CRY (cryptochrome). Also in the regulatory network are the retinoic acid-related orphan nuclear receptors REV-ERBα and RORα [118]. These molecules are present in the central nervous system as well as peripheral tissues. The impact of circadian rhythms on obesity and fatty liver disease is under active study [119]. Several master metabolic genes are regulated by the clock, including the PPAR gamma co-activators PGC1α and PGC1β and genes encoding fatty acid uptake and export [119].

Although the classical input that regulates the diurnal clock in all tissues is the availability of light, additional forms of input such as feeding can affect clocks in peripheral tissues. One intriguing observation arising from research on the circadian control of metabolism is that round-the-clock feeding of a high-fat diet induces obesity and NASH in mice, whereas the same diet given on a 12-h schedule does not, even if an identical number of calories is consumed [120]. More recently, it was reported that mice with pre-existing obesity can be cured of their dysmetabo-

lism and hepatic steatosis by simply restricting their daily access to food to a 12-h cycle per day [121]. Although more research is necessary to understand the mechanism underlying this phenomenon, the observation is likely to prompt studies in humans with NAFLD and NASH.

Pathogenesis of Liver Injury in NASH

The term NASH, in contrast to NAFLD, connotes the presence of hepatic steatosis with accompanying liver cell injury and hepatic inflammation. Depending on the severity of disease, NASH can also feature hepatic fibrosis and even liver cancer. Hepatic steatosis is at the heart of both NAFLD and NASH; consequently, there is a tendency to assume these two entities form a continuum in which disease progresses from steatosis to steatohepatitis. Indeed, this is the basis for the "two-hit" hypothesis, in which the first hit to the liver is fat accumulation and a second hit, which can take many forms, triggers liver damage [122]. Although the two-hit concept has validity, there is also reason to consider that NASH represents the consequence of multiple parallel hits to the liver [123]. In this paradigm, NAFLD does not inevitably become NASH, and NASH may not be preceded by the more benign NAFLD. The discussion that follows does not specifically enlighten this debate but does address the cellular mechanisms of liver injury in NASH. The focus is on how liver cells are damaged by lipid accumulation and how resident liver cells and recruited inflammatory cells react to this damage to stimulate inflammation, fibrosis, and cancer.

Mechanisms of Cell Death in NASH

Death Receptor Induction and Activation Death receptors, which are members of the TNF receptor superfamily, figure prominently in the pathogenesis of NASH (Fig. 4.6a). Death receptors implicated in NASH development include Fas/CD95 (Fas), the TRAIL receptor DR5, and the TNF receptor TNFR1. One of the earliest observations linking death receptors to fatty liver disease was the discovery that NASH patients express high levels of the Fas receptor on hepatocytes [124]. This was followed by the demonstration that fatty acids upregulate Fas expression by cultured hepatocytes and sensitize them to death from Fas agonists [125]. Fatty acids appear to regulate Fas through a unique mechanism involving c-Met, the receptor for hepatocyte growth factor (HGF). In normal hepatocytes, Fas and c-Met exist as a complex on the hepatocyte surface, which serves to sequester Fas from undesired activation (Fig. 4.6b) [126]. When hepatocytes are exposed to fatty acids, the Fas–c-Met complex dissociates. At the same time, fatty acids stimulate hepatocytes to produce Fas ligand (FasL); this permits an autocrine Fas–FasL interaction and promotes cell death [127]. In vivo, the importance of Fas to fatty liver disease has been documented using several independent approaches. For example, a 12-mer peptide of c-Met designed to bind and inhibit Fas protects against experimental fatty liver disease [127], whereas hepatocyte-specific deletion of c-Met, which leaves Fas uninhibited on the hepatocyte surface, enhances experimental steatohepatitis [128]. Recently, experiments have shown that the Fas receptor can also interact with the EGF receptor (EGFR) on hepatocytes. Contrary to c-Met, however, the EGFR is not complexed to Fas under basal conditions (Fig. 4.6b). Rather, the two proteins are normally separate, and fatty acids promote their interaction [129]. Insulin can signal through the EGFR to promote cell proliferation [130]. When EGFR binds Fas, however, insulin responses change from proliferative to cytotoxic. This is intriguing because it implicates insulin as a direct hepatotoxic molecule in a fatty liver.

TRAIL (TNF-related apoptosis-inducing ligand) is classically known as a tumoricidal agent [131]. TRAIL receptors, however, are also expressed on nonmalignant cells including hepatocytes, where they can stimulate cell death [132]. In the setting of fatty liver disease, the TRAIL receptor DR5 is upregulated on hepatocytes [133, 134]. Fatty acids upregulate the expression of DR5 in hepatocyte culture through

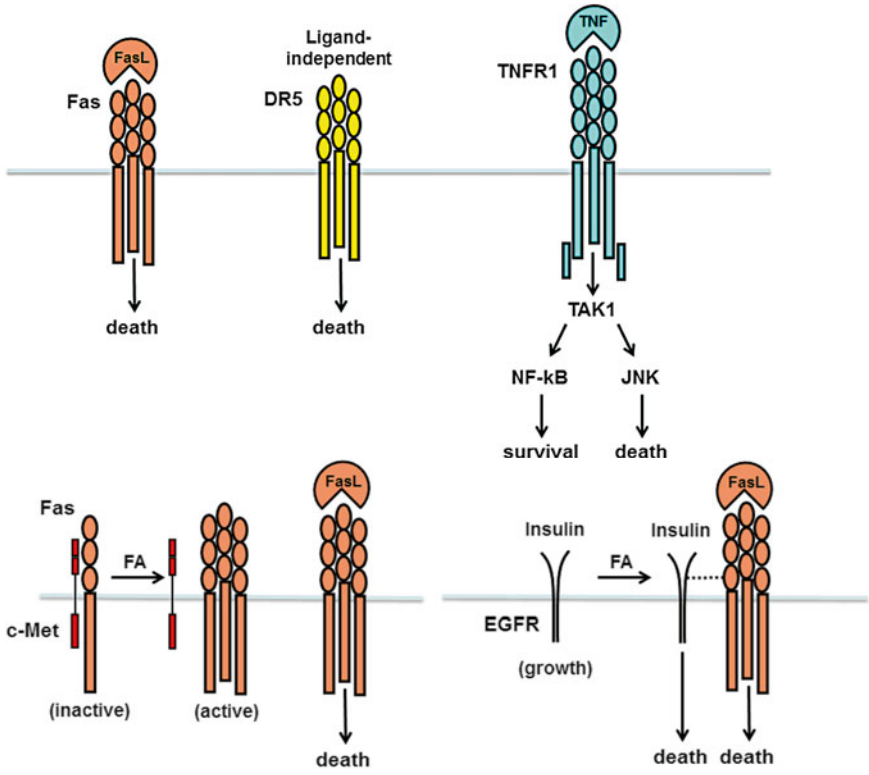

Fig. 4.6 Death receptors implicated in the pathogenesis of fatty liver disease. (**a**) Three different types of death receptors play a role in NAFLD and NASH: the Fas receptor, the TRAIL receptor DR5, and the TNF receptor TNFR1. Fas and its binding partner, Fas ligand (FasL), are both upregulated in fatty liver disease. DR5 expression is induced in hepatocytes in experimental fatty liver disease; receptor abundance leads to ligand-independent oligomerization and activation. TNFR1 can be activated on hepatocytes in a fatty liver but has the potential to signal either survival or death, via NF-κB or JNK, respectively. The outcome of TNFR1 activation depends on the balance between the two signaling processes and other simultaneous signaling processes within the cell. (**b**) The Fas receptor is subject to two unique types of regulation. First, Fas can exist in a complex with c-Met, which inhibits Fas signaling. Fatty acids stimulate dissociation of Fas from c-Met and permit cytotoxic signaling. Fatty acids can also stimulate a new association between Fas and the EGF receptor (EGFR). In this instance, EGFR signaling changes from growth to death. Thus, in the presence of fatty acids, both FasL and insulin (a ligand for EGFR) may signal cell death

a mechanism involving induction of p53 [134] or ER stress [135]. Importantly, enhanced expression of DR5 on hepatocytes can lead to cell death even in the absence of the TRAIL ligand ("ligand-independent activation"), so there is no need for an external stimulus. Hepatocytes lacking DR5 are resistant to fatty acid toxicity [135]. DR5-deficient mice also are protected against steatohepatitis in vivo, although it is currently unclear whether their improved outcome is directly attributable to reduced levels of hepatocyte death [136].

TNF is produced by dysfunctional adipose tissue [7, 8] and is present in the circulation of obese individuals [137, 138]. The cytokine is also produced locally within a fatty liver, as will be discussed below (see section "Mechanisms of Hepatic Inflammation"). Although hepatocytes express the death receptor TNFR1, TNF does not typically kill hepatocytes because the receptor signals multiple responses, not all of which lead to death (Fig. 4.6a). One of the top-level molecules activated by TNF is the MAP3 kinase TAK1 (transforming growth factor β-activated kinase 1). TAK1 in turn activates several downstream kinases, including Jun N-terminal kinase (JNK) and NF-κB. JNK activation typically promotes

cell death (see section on lipotoxicity below), whereas NF-κB activation typically promotes survival. When normal hepatocytes are treated with TNF, the NF-κB-dependent survival signals typically dominate. In situations where NF-κB responses are compromised, however, the balance is upset, and TNF induces hepatocyte death [139]. This is relevant to NAFLD because studies in mice indicate that high-fat feeding suppresses hepatic expression of TAK1 [140]. This could certainly limit the survival response to TNF, but theoretically would also reduce TNF-mediated activation of JNK. Importantly, JNK is not completely dependent on TAK1 for activation in response to TNF (see below), and thus, the balance of TNF signaling in a fatty liver could be tipped toward death.

As just mentioned, hepatocytes can be sensitized to TNF-induced death by means other than suppression of the NF-κB survival response. TNF also signals the production of reactive oxygen species (ROS), which stimulate JNK activation [141]. Consequently, any alteration that limits the control of ROS in hepatocytes could tip the balance in favor of JNK and lead to TNF-induced death. Pertinent to NASH, experimental studies have shown that diets enriched in cholesterol can compromise hepatocyte control of ROS by promoting accumulation of free cholesterol in hepatocyte mitochondrial membranes [142]. The excess cholesterol disrupts membrane fluidity, which permits the loss of an important mitochondrial antioxidant, glutathione. Notably in humans with NAFLD and NASH, free cholesterol levels are increased in the liver [117], and genes regulating cholesterol synthesis are induced [143]. Together, these observations provide a rationale for the involvement of TNF as an important mediator of cell death in NASH.

Downstream Consequences of Death Receptor Activation: Apoptosis and Necroptosis The engagement of death receptors on hepatocytes sets into motion a complex series of events that culminate in cell death. Death receptor activation can stimulate different modes of cell death depending upon the signaling pathway taken inside the cell. The classical response to death receptor engagement is apoptosis: this is a non-lytic form of cell death marked by an initial activation of caspase-8 and subsequent activation of "executioner" caspases including caspase-3, caspase-6, and caspase-7 [144] (Fig. 4.7). Death receptor activation can also induce a lytic form of death termed necroptosis [145]. In this instance, the initial intracellular event is activation of a complex between two receptor-interacting proteins (RIPs), RIP1 and RIP3. This leads to activation of mixed lineage kinase domain-like protein (MLKL), which perturbs the permeability of the plasma membrane leading to cell swelling and eventual rupture [146]. Notably, the RIP1/RIP3 complex is activated only when caspase-8 is inhibited, and thus, the two pathways are mutually exclusive within a single cell [145]. Both forms of death, however, appear relevant to NASH. In humans with NASH, activation of caspases is evident histologically and biochemically via the release of caspase-cleaved fragments of cytokeratin-18 into the circulation [147]. RIP3 is also upregulated in the livers of patients with NASH [148]. Studies in mice indicate that inhibition of caspases, either chemically or by genetic deletion, improves liver injury in experimental steatohepatitis [71, 149]. Likewise, RIP3 deletion ameliorates experimental liver injury in a mouse model of fatty liver disease [148]. Importantly, the blockade of either caspases or RIP3 alone does not completely reverse experimental steatohepatitis. This underscores that hepatocytes can follow more than one pathway to death in a fatty liver.

Death Receptor-Independent Apoptosis Apoptosis triggered by death receptor activation is considered "extrinsic." Another pathway to apoptosis, termed "intrinsic," bypasses death receptors but still culminates in the activation of executioner caspases. Certain lipids, particularly the long-chain saturated fatty acids palmitate (C16:0) and stearate (C18:0) and their downstream metabolite lysophosphatidylcholine, are capable of inducing intrinsic apoptosis of hepatocytes through a process called lipotoxicity or lipoapoptosis [150–153]. In cell culture, an important prerequisite to hepatocyte lipotoxicity

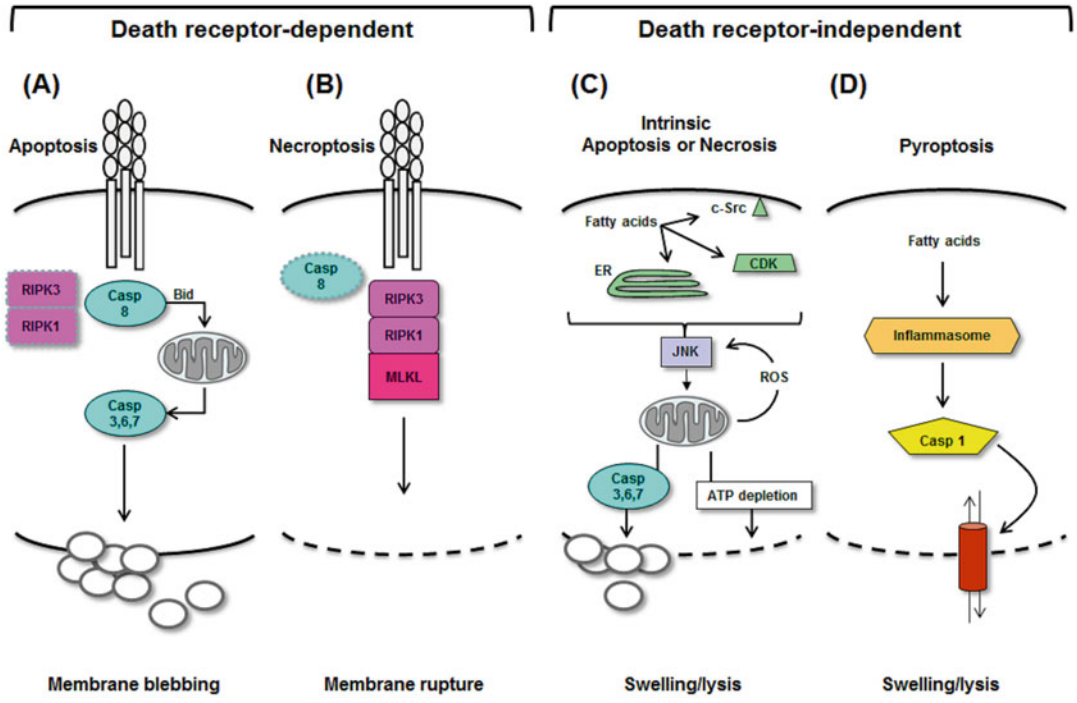

Fig. 4.7 Modes of hepatocyte death in NASH. Liver cells can take several routes to death in the setting of fatty liver disease. The modes of death can be segregated into death receptor-dependent and death receptor-independent processes. Activation of death receptors signaling either apoptosis or necroptosis. (**a**) Apoptosis occurs when death receptors activate caspase-8 (and inactivate RIP1/RIP3), leading to downstream activation of caspase-3 and condensation/blebbing of the plasma membrane. (**b**) Necroptosis occurs when death receptors are activated, but caspase-8 is inhibited, leading to downstream activation of RIP1/RIP3. This in turn leads to activation of MLKL, with subsequent lysis of the hepatocyte plasma membrane. (**c**) Fatty acids can kill cells independent of death receptors by causing intrinsic mitochondrial damage via JNK. Fatty acids can activate JNK by several mechanisms, including ER stress or activation of c-Src or cyclin-dependent kinases (CDK). JNK, once activated, binds to mitochondria and promotes ROS release. This begins a vicious cycle of JNK activation and oxidant stress that ultimately depletes cellular ATP, leading to necrosis. JNK can also cause apoptosis by stimulating mitochondria to activate caspase-3. In this case, JNK activation results in apoptosis. (**d**) Fatty acids can induce pyroptosis by upregulating inflammasomes and activating caspase-1. This opens a plasma membrane pore, leading to cell swelling and lysis

is fatty acid-induced activation of JNK [150]. In vivo as well, JNK activation is critical to the development of experimental NASH [154, 155] and has been documented in the livers of humans with NASH [117, 156]. One means by which saturated fatty acids can activate JNK is through ER stress [157, 158] (Fig. 4.7). During ER stress, JNK is activated downstream of IRE1 [159]. It then migrates to the mitochondrion where it perturbs mitochondrial respiration, prompting the release of mitochondrial cytochrome c and activation of caspase-3 [160]. JNK can also interfere with mitochondrial function indirectly, by activating pro-apoptotic proteins of the BH3-only family and inactivating their inhibitors in the Bcl-2 family, leading to a similar result [150, 161, 162]. Saturated fatty acids are also capable of activating JNK independently of ER stress. These alternative pathways involve cyclin-dependent kinases [163] or c-Src [164].

Since ER stress has been invoked as an important intermediate in fatty acid-induced cell death, it should be noted that ER stress can also lead to apoptosis through activation of PERK. PERK activation induces the expression of C-EBP homologous protein (CHOP), which in turn

stimulates the transcription of pro-apoptotic proteins and suppresses the transcription of anti-apoptotic proteins [165, 166]. Although ER stress-induced upregulation of CHOP is an important intermediate to cell death in many cell systems [167, 168], it appears dispensable to fatty acid-induced death of hepatocytes [169, 170]. CHOP also induces the expression of ER oxidoreductin-1 (ERO1), an enzyme that promotes protein oxidation in the ER and contributes to cellular oxidant stress [171]. This sensitizes cells to death, although it does not appear to intersect directly with mitochondrial dysfunction and intrinsic apoptosis.

Pyroptosis as a Potential New Mode of Cell Death in Fatty Liver Disease The term pyroptosis, which connotes a pro-inflammatory ("fiery") form of programmed cell death, was coined in 2001 [172]. Pyroptosis, like apoptosis, involves caspase activation, but is dependent specifically on caspase-1 rather than caspase-3, caspase-6, caspase-7, or caspase-8. Pyroptosis is a lytic form of cell death; it is induced when caspase-1 is activated by a multi-protein complex called the inflammasome (discussed below) (Fig. 4.7). Although the principal function of caspase-1 is to promote inflammation, it can also lead to cell death by causing pore formation in plasma membranes [173]. In the livers of experimental animals, forced activation of caspase-1 via the inflammasome causes substantial hepatocyte injury [174]. With respect to the role of pyroptosis in fatty liver disease, studies show that saturated fatty acids stimulate hepatocytes to express inflammasome components in cell culture [175] and caspase-1 is activated in experimental NASH [175, 176]; importantly, upregulation of caspase-1 has also been documented in humans with NASH [175]. Whether pyroptosis is an important mode of hepatocyte death in fatty livers is still uncertain, because caspase-1 inactivation in experimental fatty liver disease has little effect on markers of hepatocyte injury [175, 177, 176]. Recent studies indicate caspase-1 does play a role in hepatic inflammation and fibrosis in experimental fatty liver disease (see section "Mechanisms of Hepatic Fibrosis").

Mitochondrial Dysfunction and Necrosis Necrosis is an unregulated form of cell death in which profound mitochondrial dysfunction leads to depletion of cellular ATP, failure of cellular ATP-dependent ion pumps, and plasma membrane rupture [178]. A decline in mitochondrial dysfunction is an important feature of NAFLD and NASH [179], and thus, some of the hepatocyte death that occurs in fatty livers is likely due to necrosis. Mitochondrial deterioration in fatty liver disease results from sustained high levels of fatty acid oxidation; this is driven in part by the continuous influx of fatty acids from adipose tissue and in part by the need for energy to drive processes such as lipogenesis and gluconeogenesis [39, 180]. The constant flux of fatty acids through mitochondria, with attendant high-level activity of the tricarboxylic acid (TCA) cycle [181], leads to enhanced delivery of electrons to the mitochondrial respiratory chain (MRC) and electron leakage within the MRC [182, 183]. This generates harmful ROS, which can damage the components of the MRC [184] and lead to more ROS production. ROS can further exacerbate mitochondrial dysfunction by activating JNK [160]; eventually this vicious cycle will result in mitochondrial failure and ATP depletion. It is important to note that in hepatocytes, mitochondrial dysfunction is central to all types of cell death [185], and thus, distinguishing among the specific types of cell death can be difficult (Fig. 4.7). However, because of the indolent nature of NASH compared to other liver diseases defined by necrosis, such as acetaminophen toxicity and ischemia–reperfusion injury, one can deduce that necrosis is not the predominant mode of cell death in a fatty liver.

Contribution of Impaired Autophagy to Cell Death in NASH Autophagy is a process that typically promotes cell survival. Thus, any impairment of autophagy can make a cell vulnerable to death. In hepatocytes, autophagic breakdown of hepatic lipids is important for supplying hepatocyte mitochondria with fuel; consequently, any reduction in autophagy can lead to impaired mitochondrial ATP production and cell death. Impaired autophagy in a fatty liver can also lead to cell death by other means. For example,

autophagy is the process used to rid a cell of dysfunctional mitochondria [186]. Since the metabolic demand placed on mitochondria in a fatty liver leads to dysfunction, any impairment in the ability of hepatocytes to remove damaged mitochondria may lead to cell death. Impaired autophagy can also sensitize hepatocytes to TNF-induced cell death by enhancing JNK signaling and stimulating caspase activation [187]. In addition, recent work indicates there is a reciprocal relationship between autophagy and ER stress in hepatocytes [188, 189]. In this case, impaired autophagy could enhance ER stress-induced lipotoxicity. Evidence of impaired autophagy has been found in the livers of NASH patients [188, 190]. This has led to interest in testing drugs that stimulate autophagy as a treatment for fatty liver disease. Experimental data are limited but promising [191, 189].

Acidic Sphingomyelinase and NASH

Acidic sphingomyelinase (ASMase) is an enzyme that converts sphingomyelin to ceramide and phosphorylcholine. A growing body of work points to a specific role for this enzyme in the pathogenesis of NASH [192]. The ceramide generated by ASMase can facilitate TNF-induced hepatocyte apoptosis [193]; it can also inhibit the synthesis of phosphatidylcholine, which as mentioned earlier is important to hepatic lipid export as well as the integrity and function of organelle membranes [194]. Importantly, ASMase inhibits methionine adenosyltransferase [195]. This enzyme is responsible for converting methionine to S-adenosylmethionine, a critical methyl donor and glutathione precursor [196]. Thus, ASMase is integrally linked to processes pertinent to NASH, ranging from ER stress to autophagy to cell death and oxidant stress. Studies in mice indicate that genetic deletion of ASMase protects against experimental NASH [189]. Pharmacologic inhibition of ASMase with the drug amitriptyline is also effective [189], and thus, the role of this enzyme in human NASH warrants further exploration.

Mechanisms of Hepatic Inflammation in NASH

Activation of the innate immune system is a key feature of NASH. A number of different compounds can elicit innate immune responses in a fatty liver; some originate outside the liver and reach the organ from the circulation, whereas others are produced locally and act in paracrine or autocrine fashion. One external stimulus to hepatic inflammation in NASH is gut-derived bacterial endotoxin. The role of endotoxin in the pathogenesis of NASH has been established through a variety of experiments demonstrating that prebiotics, probiotics, and antibiotics, by reducing endotoxin delivery to the liver, can interfere with NASH development in experimental animals [197–199]. Second, fatty acids, which are abundant in the liver as well as the circulation in obese individuals with hepatic steatosis, can induce innate immune responses [200, 201]. Third, molecules released locally inside the liver by dead or dying hepatocytes serve as a stimulus to innate immune activation. Collectively these diverse compounds induce inflammation by acting either as PAMPs (pathogen-associated molecular patterns) or DAMPs (damage-associated molecular patterns), which engage pattern recognition receptors (PRRs) on resident liver cells.

Toll-Like Receptors in the Pathogenesis of Hepatic Inflammation The PRRs that prompt hepatic inflammation in NASH belong to the Toll-like receptor (TLR) family. Engagement of TLRs triggers inflammation by initiating a signaling cascade that activates NF-κB and culminates in the transcription of genes encoding pro-inflammatory cytokines. TLRs are expressed by all resident liver cells [202]; consequently, the liver has broad potential to respond to PAMPs and DAMPs through this mechanism. Among all the hepatic TLRs, TLR2, TLR4, and TLR9 are the ones implicated in the pathogenesis of steatohepatitis (Fig. 4.8). Traditionally, TLR2 classically recognizes peptidoglycans and other components of Gram-positive bacteria, whereas TLR4 recognizes Gram-negative endotoxin and

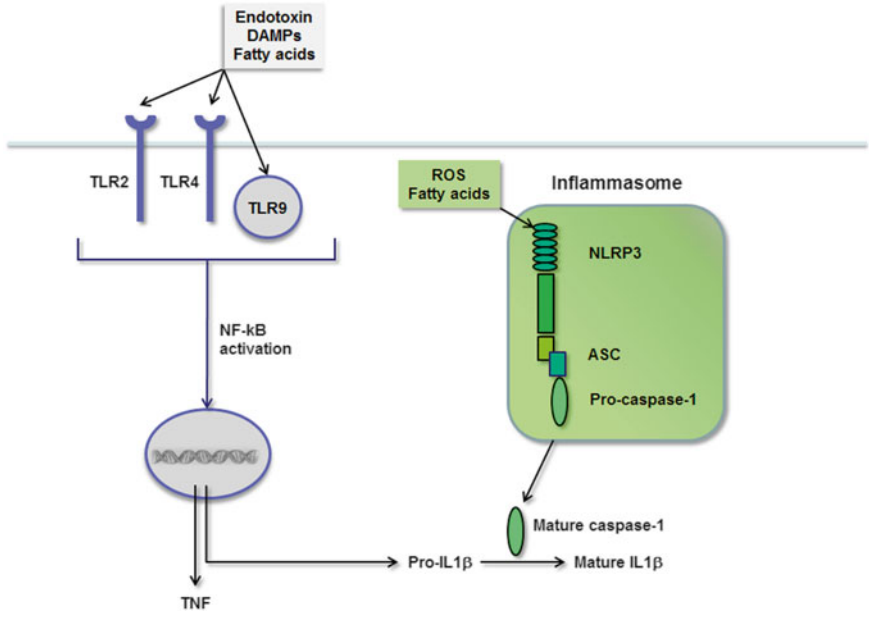

Fig. 4.8 TLRs and the inflammasome signal hepatic inflammation in NASH. TLRs are activated by fatty acids, endotoxin, and DAMPs, which leads to NF-κB activation and the production of pro-inflammatory cytokines such as TNF and IL-1β. TNF is active upon synthesis, but IL-1β is produced as an inactive propeptide. Fatty acids and ROS also stimulate the synthesis and assembly of the NLRP inflammasome, comprised of NLRP3, ASC, and caspase-1. This complex facilitates the cleavage and activation of IL-1β

TLR9 binds DNA sequences found in bacteria and DNA viruses [203]. Accordingly, it is logical that TLR4 as the endotoxin receptor would play a role in NASH-related inflammation, and mice lacking TLR4 are in fact resistant to diet-induced NASH [204, 205]. Roles for TLR2 and TLR9 are less intuitive, based on the seeming lack of appropriate ligands for these receptors. Recent data, however, indicate that TLR9 recognizes not only bacterial DNA but also mammalian DNA [206]. This makes TLR9 a potential receptor for DNA released from dying liver cells, and mice deficient in TLR9 are also protected from experimental NASH [207]. Making even better sense of TLRs in NASH is emerging evidence that TLRs 2, 4, and 9 are all capable of recognizing compounds released by injured liver cells [208]. That said, the influence of TLR2 on NASH is currently controversial. Although a role for TLR2 has been suggested based on improvement of experimental NASH in TLR2 knockout mice [209], others have observed that mice lacking TLR2 actually develop worse NASH [204]. The exact contribution of this TLR to inflammation in fatty livers is yet to be determined.

Just as endotoxin and DAMPs can bind TLRs in a fatty liver, so can fatty acids. This is particularly true of long-chain saturated fatty acids, which bear structural resemblance to the lipid A moiety of bacterial endotoxin, making them ligands for TLR4 [210]. Saturated fatty acids can contribute to hepatic inflammation in NASH by other means as well, as discussed below.

Role of the Inflammasome in Steatohepatitis As mentioned previously, TLR activation triggers inflammation by stimulating the production of pro-inflammatory cytokines. Prominent among the cytokines induced in response to TLR engagement are TNF and IL-1β. Once synthesized, TNF can be readily secreted from the cell; IL-1β, on the other hand, cannot be secreted until it is first processed from a propeptide to a mature form. The enzyme responsible for cleaving IL-1β is

caspase-1. Caspase-1 itself is initially inactive inside the cell and must be activated by proteolytic cleavage within a multimolecular complex called the inflammasome [211] (Fig. 4.8).

Inflammasomes are critical participants in inflammatory responses in a wide variety of cells and tissues. In the majority of cases, they consist of nucleotide-binding oligomerization domain (NOD)-like receptor proteins (NLRPs) that sense an activating signal, adaptor proteins termed ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain), and procaspase-1 [211]. NLRPs come in many forms, but the one capable of responding to the broadest range of activating signals is NLRP3. The cells typically equipped with inflammasome components are innate immune cells, including macrophages, neutrophils, and dendritic cells [211–214]. In the liver, Kupffer cells as resident macrophages possess all the machinery of the NLRP3 inflammasome; NLRP3 components have also been identified, however, in hepatocytes, sinusoidal endothelial cells, and hepatic stellate cells, which creates the potential for an expanded role for inflammasome complexes in liver disease. Evidence indicates that inflammasomes are associated with steatohepatitis, based on the induction of inflammasome components or the presence of active caspase-1 or active IL-1β in the livers of animals and humans with NASH [215, 207, 175, 216, 177, 176, 217, 218]. More importantly, inflammasome activation appears to directly influence the development of steatohepatitis, as targeted disruption of inflammasome components in animal models of NASH reduces IL-1 secretion and limits the severity of diet-induced hepatic inflammation [218, 177, 176].

Until recently, inflammasome-mediated cytokine activation was believed to require two independent signals: one to stimulate cytokine production and a second to stimulate assembly of the inflammasome complex [208]. Cytokine production would occur downstream of a TLR signal, whereas inflammasome activation would result from one of a number of cellular stresses. Emerging data, however, indicate that certain DAMPs can serve both functions [219, 220]. In the context of fatty liver, saturated fatty acids are in a position to singlehandedly activate IL-1β via the inflammasome. Not only can they bind TLR4 to stimulate cytokine production, but they can also activate NLRP3 by inducing intracellular oxidant stress [200, 201]. The prospect that fatty acids alone might completely activate the inflammasome is intriguing, but has not been proven experimentally. Indeed, studies to date in liver cells indicate that fatty acids do not activate the inflammasome without a second signal [175, 209]. Another provocative concept is that ER stress may act as a dual stimulus capable of stimulating IL-1β synthesis and activating the NLRP3 inflammasome [221].

Contribution of Kupffer Cells to Hepatic Inflammation in NASH Although several types of leukocytes have the potential to contribute to innate immune activation in the liver, Kupffer cells are key mediators of hepatic inflammation in NASH. These cells are uniquely positioned in the hepatic sinusoids to be the first responders to PAMPs and DAMPs, and they are robust producers of IL-1 and TNF. Kupffer cell-derived IL-1 and TNF can exacerbate hepatic steatosis by stimulating fat accumulation within hepatocytes [207, 215]; IL-1 and TNF can also kill steatotic hepatocytes and prompt the release of more DAMPs [142, 207, 222]. In addition, Kupffer cells produce chemokines such as CCL2 and RANTES (regulated on activation, normal T cell expressed and secreted) [223] that recruit inflammatory monocytes from the circulation into the liver. Collectively, therefore, Kupffer cells contribute to both the development and perpetuation of injury and inflammation in a fatty liver. Studies in mice indicate that Kupffer cell activation is critical to the initiation of hepatic inflammation in NASH. Specifically, if Kupffer cells are eliminated from the liver in the early phase of experimental fatty liver disease, cytokine production is reduced, and the recruitment of inflammatory cells to the liver is significantly attenuated [224]. Once the inflammatory process is set into motion, however, the role of Kupffer cells in NASH becomes less critical, presumably because recruited monocytes perform many of the same functions.

The mononuclear cells that invade an injured liver tend to have a more inflammatory phenotype than resident Kupffer cells [225]. Infiltrating mononuclear cells are often described as having an M1, or pro-inflammatory, phenotype as opposed to an M2 phenotype, which is anti-inflammatory [226]. M2 Kupffer cells have the ability to kill M1 cells and also to promote hepatocyte survival [227, 228]. This suggests that M2 cells are equipped to maintain homeostasis in the normal liver. M2 Kupffer cells can adopt an M1 phenotype upon activation, and the degree to which this occurs may be an important determinant of the severity of NASH. In experimental models of steatohepatitis, the severity of liver injury is often strain dependent, and this variation has been attributed to differences in Kupffer cell M1/M2 balance [229, 227]. This has generated interest in identifying factors that enhance M2 polarization; putative agents include unsaturated fats, which can activate peroxisome proliferator-activated receptor-δ (PPARδ), adiponectin, or ligands for the cannabinoid receptor CB2 [230–232].

Mechanisms of Hepatic Fibrosis in NASH

Liver fibrosis develops in NASH following a sustained period of hepatocyte injury and organ inflammation. This scenario is typical of many chronic liver diseases, and thus, it is not surprising that some of the mechanisms promoting liver fibrosis in NASH are shared with other disease processes. The hallmark of hepatic fibrosis is the priming and activation of hepatic stellate cells to a myofibroblastic phenotype. Several events can lead to stellate cell activation in fatty liver disease.

Hepatocyte Death and Liver Fibrosis One potent stimulus to stellate cell activation is the death of hepatocytes. Specifically, fragments of dead hepatocytes can be ingested by stellate cells, which triggers the transformation of stellate cells to myofibroblasts [233]. Hepatocyte death is considered an important fibrotic stimulus in fatty liver disease because pan-caspase inhibitors, which inhibit hepatocyte apoptosis, can suppress fibrosis in mice with experimental steatohepatitis [234]. Cell death may also trigger fibrosis indirectly in a fatty liver, by first stimulating hepatic inflammation through the innate immune pathways described above [71, 218]. In support of this concept is the fact that TNF and IL-1β, which are both induced in NASH-related hepatic inflammation, can promote liver fibrosis. Notably, these cytokines do not induce fibrosis by stimulating stellate cell activation, but instead facilitate stellate cell survival through the activation of NF-κB [235]. In addition, stellate cells express TLRs, which enables them to respond directly to PAMPs and DAMPs in the setting of NAFLD. In this respect, endotoxin and synthetic TLR2 ligands have both been identified as stellate cell activators. Saturated fatty acids, however, do not directly activate stellate cells [236, 209]. Overall, many of the same stimuli that promote inflammation in NASH also promote fibrosis, which may explain why fibrosis tends to improve in parallel with inflammation upon therapeutic manipulation [207, 209, 237–239].

Hepatocyte "Un-death" and Liver Fibrosis One of the hallmarks of NASH in humans is the presence of ballooned hepatocytes [240]. These cells are believed to have started down a death pathway, but stalled in the process, hence the designation "undead" [241]. Research into the phenomenon of ballooning has revealed that ballooned cells produce sonic hedgehog [242, 243, 241], a protein that promotes tissue fibrosis [244]. Hedgehog appears to mediate fibrosis indirectly by first inducing stimulating cholangiocytes to produce osteopontin. Osteopontin then acts in a paracrine fashion on stellate cells to induce fibrosis [245]. Experiments indicate that hedgehog inhibitors improve fibrosis in experimental fatty liver disease [246]), which supports the concept that hedgehog is an important contributor to NASH-related liver fibrosis. There remains some debate, however, whether hedgehog signaling specifically stimulates hepatic fibrosis or plays a role in upstream disease processes as well, such as hepatocyte death [247].

Influence of Adipokines on Liver Fibrosis Some compounds with relevance to obesity and the metabolic syndrome have unique influences on stellate cells. Leptin, for example, which is abundant in obesity, interacts directly with HSC to stimulate collagen production [248]. Adiponectin, by contrast, which is scarce in individuals with the metabolic syndrome [249], suppresses stellate cell activation [250]. Thus, the adipokine profile of patients with NAFLD/NASH is one that favors hepatic fibrogenesis. Other adipokines, including resistin, visfatin, and apelin, correlate positively with NASH and liver fibrosis, but their roles in stellate cell activation are less well defined (reviewed in [251]).

Hepatocarcinogenesis in NASH

Just as chronic liver injury predisposes to hepatic inflammation and fibrosis, it also facilitates the development of liver cancer. A recent review [252] provides an excellent overview of the signaling pathways relevant to hepatocarcinogenesis in NASH. Observations in experimental animals have highlighted a role for NAFLD as an accelerant to liver cancer; specifically, they have shown that a carcinogenic insult is much more potent when administered on a background of obesity and fatty liver [253–255]. In one instance, mice fed a high-fat diet and then treated with the carcinogen diethylnitrosamine (DEN) developed liver tumors of twice the number and twice the size as mice given DEN with a standard diet [255]. Two factors implicated in the acceleration of tumor growth in the obese mice were IL-6 and TNF. These cytokines, which are both known to be upregulated in obese mice, enhanced hepatic activation of the stress kinases JNK and ERK and accentuated hepatic steatosis and inflammation in response to DEN.

The discovery that obesity and fatty liver enhanced JNK activation in the livers of obese DEN-treated mice is of interest because liver cancer arises in other animals that exhibit an imbalance between JNK (death) signaling and NF-κB (survival) signaling in hepatocytes in response to TNF [256] (Fig. 4.6a). More recently, researchers discovered another important driver of hepatocarcinogenesis downstream of TNF, mTOR. As mentioned earlier (see section "Death Receptor Induction") JNK and NF-κB are activated by TNF through the MAP3 kinase TAK1. Importantly, TAK1 also activates AMP kinase, which then phosphorylates and inactivates mTOR [257]. In genetic and diet-induced obesity, however, TAK1 is suppressed [140]; this leads to abnormal mTOR activation, with an attendant increase in hepatic lipogenesis, decrease in hepatocyte autophagy, and decrease in fatty acid oxidation. In the extreme case of genetic ablation of TAK1, high-fat feeding induces steatohepatitis, hepatic fibrosis, and tumor formation, all of which are inhibitable by the mTOR inhibitor rapamycin. This implicates mTOR as an important driver of hepatocarcinogenesis in conjunction with JNK/NF-κB imbalance [140].

Yet another intriguing finding from a recent animal study is that ER stress in hepatocytes predisposes to liver cancer. What made this study unique was that ER stress was induced only transiently in mice, before the initiation of a high-fat diet. The mice that underwent, but then recovered from, an initial ER stress subsequently developed accentuated ER stress in response to high-fat feeding. This resulted in greater hepatic steatosis, more cell death, and more liver tumors [258]. This raises the possibility that a previous hepatic insult sensitizes a liver to metabolic damage, including liver cancer. In this respect, prior ER stress can serve as a risk factor for enhanced liver damage, rather than a preconditioning event that would suppress subsequent injury.

Summary

As knowledge about cell biology advances, so does our concept of the pathogenesis of fatty liver disease. This is immediately evident in the fact that we now include ER stress, autophagy, and inflammasome activation among the pathways contributing to hepatic steatosis and NASH. These new paradigms are not replacing old theories; rather, they are putting them into new perspective and in many cases permitting consolidation of

seemingly unrelated events into single pathogenic schemes (e.g., consider fatty acids, dead cells, and endotoxin all as inducers of the inflammasome in a fatty liver). Although for simplicity, specific disease mechanisms were often addressed individually in this chapter, they frequently intersect. For example, there is crosstalk between ER stress and autophagy [259], and JNK activation can result from numerous insults including ER stress, death receptor signaling, and ROS induction. This underscores the true complexity of disease pathogenesis in a fatty liver.

With regard to the pathophysiology of NASH and its complications, one theme that emerges from the above discussion is the central role of hepatocyte death in the process. Indeed, hepatocyte death triggers not only inflammation but also fibrosis and even cancer. With this in mind, it remains a conundrum why some individuals at risk for fatty liver disease develop only hepatic steatosis, whereas others develop NASH. Further exploration of genetic and behavioral modifiers is likely to shed light on this issue.

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Animal Models of Alcoholic Liver Disease

5

Jun Xu and Hidekazu Tsukamoto

Conceptual Requirements for Animal Models of ALD

For an animal model to serve as a useful experimental tool for research on a disease of interest, it has to meet fundamental requirements. Table 5.1 enlists such requirements for an optimal model of ALD. First and utmost, a model has to faithfully reflect etiological background and natural history of a disease seen in patients. Clinically important spectra of ALD are chronic alcoholic steatohepaitis (ASH), alcoholic hepatitis with neutrophil infiltration (AH) on the background of chronic ASH, and cirrhosis. These pathologies are caused by heavy drinking, not by moderate drinking, driven by addiction to ethanol. This is a simple but yet important requirement we needed to be reminded of. Alcoholics who have physical dependence on ethanol need to titrate throughout the day their ethanol intake to maintain blood ethanol concentrations (BACs) above "threshold" levels to avoid unpleasant withdrawal symptoms. These threshold BACs are often raised in alcoholics due to metabolic and physical tolerance, driving a vicious cycle of enforcing increased and sustained ethanol intake—a definition of alcoholism. Indeed, AH and cirrhosis are associated with continuous or steady drinking rather than frequent or episodic drinking pattern [1, 2]. Thus, an animal model of ALD has to reproduce this salient feature.

ALD is the disease which develops after 15–20 years of heavy drinking in man and 10–15 years in woman. This chronicity is an important consideration and means that in rodents, for example, 2–6 months of drinking in the quantities that maintain sustained BACs, must be required. The pattern of ethanol intake is also critical. As already discussed above, alcoholics continually take ethanol throughout the day to maintain BACs every 3–4 h between drinking episodes from morning through evening followed by a large dose before they sleep. Binge drinking pattern is commonly observed among youth [3], and binge superimposed to steady heavy drinking is frequently associated with AH [4, 5]. The model needs be able to test this chronic plus binge pattern. The model should also allow incorporation of genetic and environmental risk factors known for ALD patients [6]. This is particularly essential as research in the past 4 decades has led to a consensus that heavy ethanol

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Table 5.1 Requirements for ALD animal models

1.	Heavy and steady ethanol intake
2.	Physical and metabolic tolerance to ethanol: achieving high BACs which are tolerated by the model
3.	Chronicity of ethanol intake: at least 10~25 % of life span
4.	Incorporation of genetic and environmental risk factors
5.	Reproduction of clinically relevant, advanced ALD liver pathology
6.	Reproduction of clinical symptoms/complications of ALD patients (portal hypertension, coagulopathy, hypoalbuminemia, hyperbilirubinemia, muscle wasting)

intake is required but not sufficient alone to cause advanced ALD. Lastly, the model should reproduce not only advanced ALD spectra but also symptoms and complications associated with them, such as endotoxemia, bacteremia, jaundice, portal hypertension, and ascites, which are clinically and therapeutically important in dealing with ALD patients. Only then, the model will be realistically useful for understanding the mechanisms underlying complications and testing new therapeutic modalities. This review serves to critically analyze the animal models of ALD from the viewpoint of these requirements.

Historical Perspective

History repeats itself. Much can be learned from it to move forward and avoid unnecessary repetitions. This principle also applies to science and certainly for research on animal models of ALD. By reviewing the history, we realize that there are key messages to learn from the contributions made in the past. Historical descriptions summarized below are in part based on excellent reviews previously published [7–9] and will touch upon major developments and directions the field has experienced since 1930 (Fig. 5.1).

Earlier Attempts of Ethanol Gavage for Forced Administration

As early as the 1930s, investigators realized that animal's natural aversion to ethanol is a major limiting factor, necessitating the use of stomach tube for ethanol administration—gavage ethanol administration. Connor and Chaikoff fed dogs a high-saturated-fat, high-protein diet (lard and lean meat) along with vitamin and salt mixtures for 30–35 days to generate fatty liver and then gave lean meat ad lib and ethanol via gavage (approx. 2 ~ 3 g/kg) twice daily for 4–7 days. This regimen was alternated with a similar period of feeding the lard/meat diet for up to 106 days. All 16 dogs that underwent this treatment developed severe fatty liver with hyaline degeneration, and 4 of 16 dogs showed cirrhosis resembling early fatty cirrhosis in man. Although an appropriate control group was missing, this study most likely was the first 2-hit experiment with high fat and ethanol [10]. Connor then took a similar approach of feeding a diet high in protein and fat supplemented with vitamins to rabbits and administered ethanol via gavage for 3–8 months. Some had developed cirrhosis which appeared to have resulted from portal-portal bridging fibrosis [11]. A common pitfall of these earlier studies was poor nutritional status as ethanol intoxication achieved by gavage prevented the animals from having normal feeding. Chey et al. performed a more controlled study by feeding dogs a beef diet and ethanol via surgically implanted gastric fistula 5 days a week for 10–18 months achieving sustained nutrition and BACs (315–477 mg%). Liver histology showed fatty liver, ballooned hepatocyte degeneration, zonal and spotty necrosis, inflammation, and centrilobular fibrosis, the changes comparable to those seen in pre-cirrhotic ALD in patients [12].

Rodent ALD Model: A Debate Over Nutritional vs. Hepatotoxic Disease

In the comprehensive review by Moon in 1934, he pointed out that the literature lacks evidence to support that ethanol is a direct cause of liver damage [13]. This was followed by the findings that rats given 20 % of ethanol in drinking water developed cirrhosis when they were fed a low-protein, low-choline diet but not when these dietary deficiencies were corrected by supplementations [14–16]. These results supported the

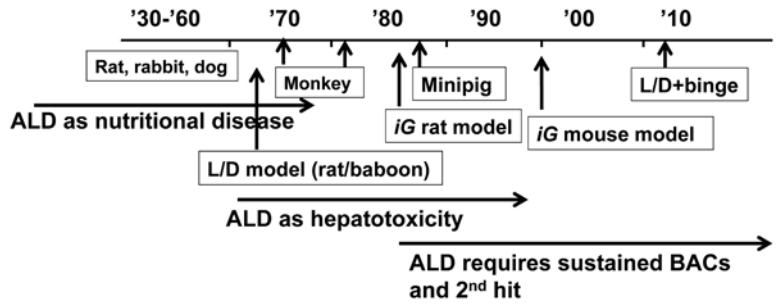

Fig. 5.1 Historical perspective. A schematic diagram depicting the times when different models were developed or used and different proposals were made for ALD pathogenesis. For simplicity, only selected models and hypotheses are highlighted

notion first proposed by Kennedy in 1933 that dietary deficiency was the crucial etiological factor in ALD [17]. Ashworth conducted the first rat model study utilizing forced ethanol intake by stomach tube and ad lib diet feeding and concluded that fatty liver produced in the model was a direct toxic effect of ethanol [18]. But as in the rabbit studies described above, poor feeding and nutrition caused by ethanol gavage was problematic. In 1949, Best et al. introduced for the first time isocaloric pair feeding by substituting ethanol consumed by ethanol diet-fed mice with equal calories of sucrose. Ethanol-fed mice developed fatty liver, but it was prevented by supplementation with a methyl donor such as methionine, choline, or casein [19]. This finding supported the concept that ethanol-induced fatty liver is caused not by ethanol's toxic action but by increased requirements for choline and other nutrients by ethanol. Subsequent studies substantiated this concept. In particular, a series of publications by Porta and Hartroft established the importance of lipotrope deficiency in exacerbation of ALD and induction of cirrhosis [20, 21]. Methionine- and choline-deficient diet commonly used for NASH studies now originated from these earlier studies that established the importance of the deficiency of these nutrients in promoting alcoholic cirrhosis. In 1963, Lieber et al. developed an ethanol-containing liquid diet which contained all required nutrients in adequate concentrations and a control diet in which ethanol was isocalorically substituted by carbohydrate [22]. This diet achieved ethanol intake of 12–18 g/kg/day, 36 % of caloric intake from ethanol, and postprandial BACs of 100–150 mg%, in young growing rats (1 month old weighing ~150 g). Fatty liver still developed in these rats, supporting the view that ethanol exerts toxic effects to cause liver pathology despite adequate nutrition [22, 23]. This hepatotoxicity hypothesis was mechanistically supported by the MEOS (microsomal ethanol-oxidizing system) that the Lieber laboratory described [24, 25] and was later identified to be CYP2E1 and CYP reductase [26, 27]. However, the Lieber-DeCarli (L/D) diet method was criticized for not providing adequate nutrition and optimal growth in growing rats [28]. This nutritional inadequacy resulted from limited intake of ethanol-containing diet due to the animal's aversion to ethanol, equally limiting the intake of control diet by pair-fed control rats. Thus, even with adequate concentrations of macro- and micronutrients in the diet, intake of suboptimal quantities of the diet resulted in marginal nutritional status. In support of this notion, if ethanol content in the L/D diet was reduced to 26 % of calories from 36 % calories (the diet with more diluted ethanol), the volume of the ethanol diet consumed by rats increased to 50 %, still achieving comparable daily ethanol dose per mouse compared to the 36 % diet; the rats grew optimally [29], and alcoholic fatty liver was prevented [30].

Subhuman Primate Models

The nutrition vs. toxicity debate also involved primate models. The L/D diet was applied to baboons, and as they consumed as much as 50 % of calories from ethanol over 1–4 years, they developed fatty liver, steatohepatitis, perivenular and bridging fibrosis, and cirrhosis [31, 32].

A primary conclusion from these studies was that baboons tolerated higher ethanol intake and developed progressive ALD while rats given L/D diet had limited liver pathology due to lower ethanol intake and BACs. However, *Macaca radiata* monkeys [33], rhesus monkeys [34], and baboons [35] fed with L/D or similar diet did not develop advanced ALD as observed in Lieber's studies.

Miniature Pig Model

Hanford miniature pigs have been fed low- or moderate-fat (5 % Cal or 12 % Cal) regular chow mixed with ethanol (4 g/kg/day, 40 % Cal) for 12 or 20 months to study alcoholic liver injury. Under these regimens, the average BAC levels of 130–229 mg% were measured; serum transaminase levels were modestly elevated, but liver histology was not altered [36]. However, when the corn oil content in the diet was increased to 33 % Cal in Yucatan miniature pigs, ethanol feeding resulted in fatty liver and liver fibrosis [37]. Two major advantages of this model are that (1) miniature pigs have anatomical, physiological, and immunological similarities with human and (2) miniature pigs have the voracious appetite with less aversion to ethanol or dietary manipulation as compared to other small animal models. Thus the model has the potential for further experimental manipulations including incorporation of the second or multiple hits that may lead to reproduction of advanced ALD. Obvious downsides of the model are longer periods of feeding and higher animal and per diem costs.

Sustained BACs by Intragastric Feeding (iG) Model

One hypothesis which emerged from the studies on the rat and primate models described above was the importance of sustained BACs in the ALD pathogenesis. This was a difficult requirement to fulfill in animal models. Animals with aversion to ethanol do not become sufficiently addicted to self-administered large quantities of ethanol to the degree seen in alcoholics, and animals have higher basal metabolic rates which directly translate to faster ethanol metabolism, making sustained and high BACs in animals especially in rodents almost impossible. This challenge served as an impetus for the development of intragastric ethanol feeding model in rodents. The iG model allowed excessive ethanol intake, sustained BACs (250–350 mg%), and controlled nutritional intake [38]. In adult rats, iG model achieved ~49 % Cal intake from ethanol and induced severe fatty liver even with a low-fat diet [38]. More advanced pathology such as liver necrosis and fibrosis was produced with high-fat diet [39] and cirrhosis with a combination of high-fat diet and iron [40], thus supporting the importance of both sustained BACs and the secondary risk factors in progressive ALD. In the 2000s, Thurman's laboratory has performed a series of elegant mouse iG experiments to demonstrate the importance of CD14, ICAM-1, TLR4, and LBP in experimental ALD by performing genetic loss of function experiments [41–44]. This mouse iG model has evolved in the past 2 decades (Fig. 5.2) [45] and revealed the importance of clinically relevant 2nd hit such as hepatitis C virus protein expression [46] and obesity [47] which will be discussed below.

Three noteworthy observations were made in the iG models concerning their BACs and metabolic and physical tolerance. Firstly, the animals exhibited unexpected cyclical daily BACs with a magnitude of 50–450 mg% peaking every 5–6 days despite continuous iG infusion of a constant ethanol dose (Fig. 5.3a) [48]. This cycle was triggered by induction of ethanol clearance with the threshold BAC of ~250 mg%. This phenomenon was confirmed independently by other laboratories [49, 50] and mimicked the cyclical pattern of the amount of ethanol consumed by monkeys with iG self-administration (Fig. 5.3b) which simulated the pattern of ethanol consumption by alcoholics [51]. In the iG model which bypasses the cephalic and oral phases of intake, the rate of ethanol consumption is most directly governed by the rate of ethanol metabolism. Thus the exhibition of the cyclic BAC pattern must be metabolic in origin. If ethanol metabolism peaks every 5–6 days in man, this may underlie binge

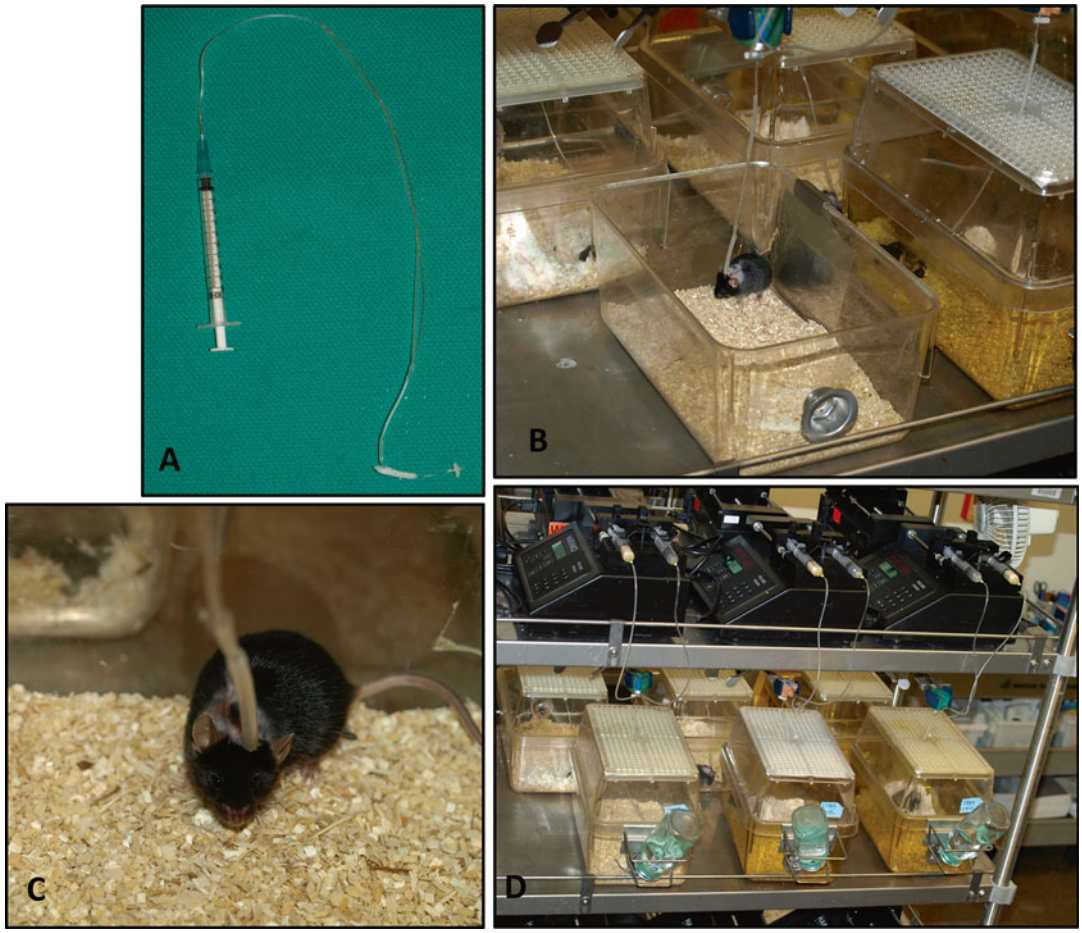

Fig. 5.2 Mouse iG model. (**a**) The *iG* catheter used for the model. (**b**) An *iG* mouse is placed within an individual micro-isolator cage. The catheter is connected to a flow-through swivel clamped above the cage. (**c**) A close-up view of the *iG* mouse. (**d**) An overview of the *iG* model setup

intake with a similar interval seen in alcoholics. The second observation was remarkable metabolic tolerance that the iG rodents underwent. The progressively increasing dose of ethanol was tolerated by these animals, depicting their remarkable metabolic reserve which was only unmasked by the iG method. This metabolic tolerance is matched by physical tolerance. Under the iG regimen, the BAC of 250 mg% or less does not cause any noticeable physical sign of intoxication. At the cyclical peak of BACs of 450–550 mg%, the animals exhibit suppressed motor coordination, particularly in lower part of the body, and only when BACs exceed 600 mg%, anesthetic effects of intoxication ensue. This remarkable physical tolerance develops because of heavy ethanol intake achieved in the iG model. Therefore, it seems that ethanol intake and intoxication facilitated by ad lib feeding methods reflect only mild level of drinking which does not reproduce heavy drinking of ALD patients.

Chronic Ethanol Plus Binge

Stomach gavage has been used since the 1930s as the method of force-feeding ethanol to animals that otherwise consume less due to the aversion. When binge is given subsequently to 10 days of L/D diet feeding, neutrophilic infiltration is

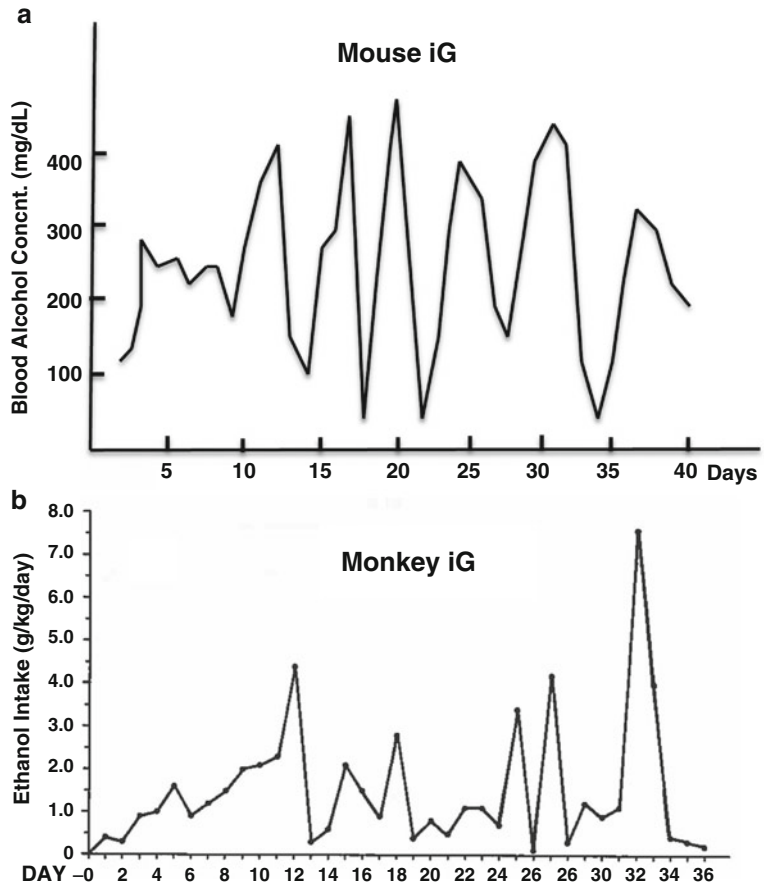

Fig. 5.3 Cyclical phenomenon. (**a**) Cyclic blood ethanol concentrations of an *iG* mouse despite the constant ethanol infusion. (**b**) A similar cyclic pattern is observed in the dose of ethanol consumed by a monkey who self-administered it via *iG* catheter (A figure with permission from Altshuler, H.L., Intragastric self-administration of ethanol: a subhuman primate model of ethanolism. In: Animal Models in Ethanol Research. Ed., Eriksson K, Sinclair JD, Kiianmaa K. Academic Press, New York, 1980, pp179–183.)

induced with elevated serum ALT [52, 53]. This rather simple model, termed "NIAAA model" [52], has been used to disclose the importance of E-selectin and invariant natural killer T cells in neutrophilic inflammation [53, 54]. Binge can also be repeated as seen in alcoholics, and this approach combined to the iG mouse model fed with Western solid diet ad lib resulted in alcoholic neutrophilic hepatitis in mice with clinical features of AH in patients [55] as discussed in detail below.

Comparisons of Different Rodent ALD Models

Rodents are convenient and common species used for ALD research. For this reason, innovations that make rodent ALD models more clinically relevant, useful, and versatile are important in the field. We have witnessed such developments in recent years and this section will highlight them in addition to providing a generalized comparative summary on the procedures, liver pathology outcome achieved and not achieved, and advantages vs. weaknesses of different rodent models used in the past and currently (Table 5.2).

Acute and Subacute ALD Models

Administration of ethanol through oral gavage or intravenous injection for a short period has been used to evaluate acute (up to 72 h) and subacute (up to 7 days) effects of ethanol exposure. The simplest method for acute ethanol exposure is single binge (oral gavage) with 4–7 g/kg body

Table 5.2 Rodent models available for ALD research

EtOH administration	Models	Duration	Species	ALT & AST (U/L)	Liver pathology				Comments and references
					Steatosis	Necrosis	Inflammation	Fibrosis	
EtOH in drinking water, ad lib	20–40 % (v/v)	7–29 w	Mouse Rat	ALT: ~68 AST: ~107	++	+	++	−/+	• Convenient but long duration • Moderate liver injury seen in rat [56] not in mouse [57–59]
Oral gavage (EtOH in water, 32–50 %, v/v)	Acute single binge (4–7 g/kg)	4–72 h	Mouse	ALT: 4 h: ~116 12 h: ~74 24 h: ~31 72 h: ~32	ND	ND	ND	ND	• Acute and transient elevation of ALT [60] and LPS [61] • mtDNA depletion [62, 63]
	Two-hit model: acute single binge + LPS (5 mg/kg, i.v.)	24 h	Rat	AST: ~779 ALT: ~760	−	++	++	−	• Neutrophil infiltration • EtOH causes early tolerance and late sensitization of KC to LPS [61]
	Subacute repeated binge (4–5 g/kg, every 12–24 h)	3–4 d	Mouse Rat	ALT: ~45	−/+	−	−	−	• MtDNA depletion and impaired hepatic respiration [64]
	Two-hit model: subacute repeated binge in obese rat	3 d	Rat	ALT: ~125	+	−	+	−	• Obesity aggravates liver injury through oxidative and nitrosative damage [65]
	Chronic repeated binge (5 g/kg daily)	8 w	Rat	ALT: ~120 AST: ~160	+	+	+	−	• Elevation of LPS and KC activation [66]
Intravenous	Single i.v. injection (1.75 g/kg) followed by i.v. infusion (250–300 mg/kg/h)	18 h	Rat	ALT: ~115	ND	ND	ND	ND	• Superoxide-generated post-acute EtOH exposure contributes to liver injury [67] • Cross-tolerance between EtOH intoxication and endotoxemia [68]
Lieber-DeCarli, (L/D) diet	Ad lib feeding	10 w–9 m	Mouse Rat	AST: ~120	+	−	−/+	−	• Convenient • Do not represent human ALD [23, 69]

(continued)

Table 5.2 (continued)

EtOH administration	Models	Duration	Species	ALT & AST (U/L)	Liver pathology				Comments and references
					Steatosis	Necrosis	Inflammation	Fibrosis	
	Two-hit model: ad lib L/D diet + LPS i.v./i.p. (0.5–1 ug/g)	10 w	Rat	ALT: ~400 AST: ~500	++	+	+	−	• Some features of human AH [70, 71] HSC activation [72]
	Two-hit model: ad lib L/D diet + CCl4 (1 μl/g)	3–5 w	Mouse Rat	ALT: ~350 AST: ~340	+	−/+	+	−/+	• Early signs of hepatic fibrosis [73, 74]
	10-day ad lib L/D + single EtOH binge (5 g/kg)	10 day	Mouse	ALT: ~250 AST: ~400	+	−	+	−	• Mild and transient neutrophil infiltration [53]
Western ethanol diet plus weekly binge	Ad lib feeding of liquid diet high in cholesterol and saturated fat plus weekly ethanol binge (3.5–4.5 g/kg)	8 w	Mouse	ALT: ~100	+++	++	+++	+++	• Easy procedure • Control mice also develop mild fatty liver • Activated hepatic stellate cells from the model have been characterized [75]
Chronic intragastric infusion (iG)	Standard iG: EtOH (8–12 g/kg/day)	10–16 w	Rat	ALT: ~460 AST: ~446	++	+	++	+	• Sustained high blood alcohol levels • Mononuclear cell infiltration and activation [38, 45]
	Standard iG: high dose EtOH (27–32 g/kg/day)	7–8 w	Mouse	ALT: ~250	+++	+	++	+	• Show EtOH dose-dependent liver injury [47]
	Two-hit model: iG + enteral LPS (5 mg/kg, weekly)	9 w	Rat	ALT: ~227	++	++	+++	++	• Two-hit model • Enteral LPS challenge potentiate liver injury [76]
	Two-hit model: iG + iron dextran (~75 mg/kg, s.c.)	4–16 w	Mouse Rat	ALT: ~500 AST: ~290	+++	++	+++	+++	• Two-hit model • Fibrogenesis and cirrhotic change [40, 77]
	More advanced ALD iG models	Please refer to Table 5.3 for details							

AH alcoholic hepatitis, *iG*: intragastric infusion, *KC* Kupffer cells; *L/D* Lieber-DeCarli diet, *ND* not determined; *h* hours, *d* days, *w* weeks, *m* months

weight of ethanol. Binge elicits physiologic and biochemical responses to acutely raised BACs including oxidant stress [67], lipolysis [78], endotoxemia [68, 79], and hepatic microcirculation response to transplantation [80] and acetaminophen [81]. Binge suppresses both intestinal fatty acid oxidation and triacylglycerol synthesis [82], which lead to epithelial damage. Binge also causes a swift increase in ethanol metabolism (SIAM) [83] and mitochondrial DNA (mtDNA) depletion [62, 63]. SIAM is associated with significant increase in hepatic oxygen consumption, altered glucose/lipid metabolism, activation of Kupffer cells, and pericentral hypoxia [83, 84], which are responsible for production of cytokines and prostaglandins and subsequent hepatocyte damage [85]. Acute single binge also depletes mtDNA by 51 % after 2 h of ethanol administration, and this effect is prevented by 4-methylpyrazole, an inhibitor of ethanol metabolism, and attenuated by melatonin [62, 63]. Thus, acute single binge causes SIAM in the liver, resulting in oxidative stress, mtDNA depletion, and subsequent early liver injury. It is noteworthy that binge drinking also increases serum endotoxin and bacterial DNA levels in healthy individuals, supporting the clinical relevance of the observations made in animals [86].

Although acute single binge causes mild and transient increase in levels of BACs, serum ALT, and LPS, it produces minimal pathological change in liver [60, 61]. When oral binge is given every 12 h or 24 for 3–4 days, there is little liver pathology observed [64, 66]. A significant increase in serum ALT and mild liver pathology are induced when these binge models are co-administrated with LPS [61] or binge is given in obese animals [65] (two-hit models). For example, acute single binge followed by LPS (5 mg/kg, i.v.) causes a surge of serum ALT levels up to 760 U/L and submassive necrosis and neutrophil infiltration [61].

In conclusion, although the acute and subacute ALD models are convenient and have no or minimal mortality rate, they do not produce classic features of human ALD. However, they are useful for mechanistic evaluation of ethanol action, early phase of liver injury, and ethanol's priming or sensitizing effects on hepatic responses to the second hit.

Chronic ALD Models

Chronic Binge Model Enomoto et al. demonstrated time-dependent liver injury in rats by daily oral gavage of ethanol (5 g/kg body weight) for 8 weeks [66]. Mild steatosis and mild ASH were observed at 4 weeks and 8 weeks after daily binge, respectively. The authors further demonstrated that sensitization of Kupffer cells to LPS contributed to ethanol-induced liver injury. However, they noted that repeated binge also result in endotoxin tolerance depending on the duration of the treatment [87]. This model is simple but daily binge for such a long period requires high skill of oral gavage, and only limited liver pathology is achievable by the method.

Ethanol in Drinking Water Model Feeding rodents ethanol in drinking water is the simplest method. Rats with 40 % (v/v) ethanol in drinking water for up to 29 weeks developed mild to moderate ASH and some fibrotic changes in the liver [56]. However, mice given 20 % (v/v) ethanol in drinking water for 21 weeks did not have significant elevation of serum ALT and histology showed mild hepatic steatosis [57–59]. Mild liver pathology produced despite the prolonged treatments and the lack of nutritional control are major limitations of this method.

Chronic Lieber-DeCarli Liquid Diet (L/D) Model Ad lib feeding ethanol-containing L/D diet is widely used in the research field of ALD because it allows easy ethanol administration and dietary modification. As described above, baboons fed L/D diet twice daily for 22 months resulted in severe liver damage similar to that of human alcoholics, including inflammation, fibrosis, and cirrhosis [32]. However, rodents fed the L/D diet for up to 10 weeks develop only mild hepatic steatosis [23, 69, 70]. Hepatic injury beyond steatosis is rare even when rats are fed L/D diet for up to 9 months on ad lib feeding [88]. As discussed earlier, this is due in part to animal's aversion to ethanol, resulting in only moderate BACs being reached by ad lib L/D feeding [89]. Advanced liver injury in L/D diet-fed rodents develops when the second insult is delivered. For example, rats fed L/D diet for

10 weeks followed by single i.v. injection of LPS develop focal necrosis and inflammation [70, 71] and hepatic stellate cell activation [72], while rats receiving LPS treatment on control diet develop no or minor liver lesions. Similarly, rodents fed L/D diet followed by CCl4 treatment develop inflammatory and fibrotic changes in the liver as compared to the L/D diet-fed animals [73, 74]. In these models, the L/D diet serves to promote LPS- or CCl4-mediated liver injury, and liver pathology produced reflects heightened damaging processes and patterns of the second hit, rather than alcohol. The L/D liquid diet model allows easy manipulation of diet components, including lipids, proteins, or other dietary constituents. This method is thereby suited for studies on how moderate ethanol intake primes or sensitizes the liver to injurious effects caused by the secondary hit or by interactions with nutritional modifications. The L/D diet with reduced ethanol concentration can be given for an extended period of 10–12 months to promote liver cancer development in HCV transgenic mice [46].

10-day L/D Diet Plus Binge As briefly described above, Bertola et al. developed a mouse model by combining the L/D ethanol liquid diet feeding with acute ethanol binge, commonly called chronic binge or NIAAA model [52, 53]. The model is based on 10 days of L/D ad lib feeding and one single binge and produces elevation of serum aminotransferases, hepatic cytokine expression, and neutrophil infiltration peaking at 9 h followed by normalization at 24 h [52]. This simple and easy model which achieves a snapshot of liver injury with inflammation provides the field a useful tool for mechanistic studies targeted to the convenient window of time. Limitations include the transient nature of liver injury which does not mimic advanced ALD, requiring validation of findings in more chronic models of ALD.

Western Ethanol Liquid Diet With or Without Weekly Binge The field has long desired an easy animal model which produces chronic ASH and alcoholic liver fibrosis. To this end, we have developed an ethanol-containing Western liquid diet with two different concentrations of cholesterol and saturated fat in collaboration with Dyets, Inc. (www.Dyets.com). One (DYET#710142) contains cholesterol (0.23 % w/w), lard (20.9 % Cal), anhydrous milk fat (12.5 % Cal), and corn oil (4.2 % Cal). In another diet (DYET#710384), lard and cholesterol contents are reduced by half and corn oil doubled (reduced saturation). Using these diets, ethanol and Western diet can be given ad lib to mice in a manner identical to L/D diet. Although ethanol intake is still limited and BACs attained are low as with L/D diet, they provide the ethanol Western diet two hits. Liver pathology produced by this Western ethanol liquid diet is still limited to fatty liver with mild increases in serum ALT. Further, mice given control diets also develop mild microvesicular steatosis, although the reduced cholesterol/lard diet attenuates this effect. However, if these diets are combined with weekly ethanol binge starting with 3.5 g/kg and increasing to 4.5 g/kg over 8 weeks, ASH with neutrophilic infiltration and perisinusoidal liver fibrosis develops (Fig. 5.4b, c), and serum ALT is significantly increased (Fig. 5.4d). Hepatic stellate cells isolated from the model are activated with *Col1a1* and *Timp1* upregulation (Fig. 5.4e), and epigenetic changes such as H3K4me3 enrichment are noted genome wide [75]. A drawback of this model is that as ethanol intake is limited, the model relies relatively more on the second hit, resulting in the mice fed control Western diet developing fatty liver as an obvious diet effect (Fig. 5.4a). Western ethanol liquid diet without binge also serves as an excellent tumor promoter for DEN-initiated liver tumorigenesis in mice. With this diet, aggressive liver tumors develop within 4–5 months following DEN injection at 2 weeks of age in male C57/B6 mice without phenobarbital in drinking water.

iG Model: Different Versions

The iG model, particularly mouse model, has evolved substantially during the past 15 years. Our center's animal core presently produces four

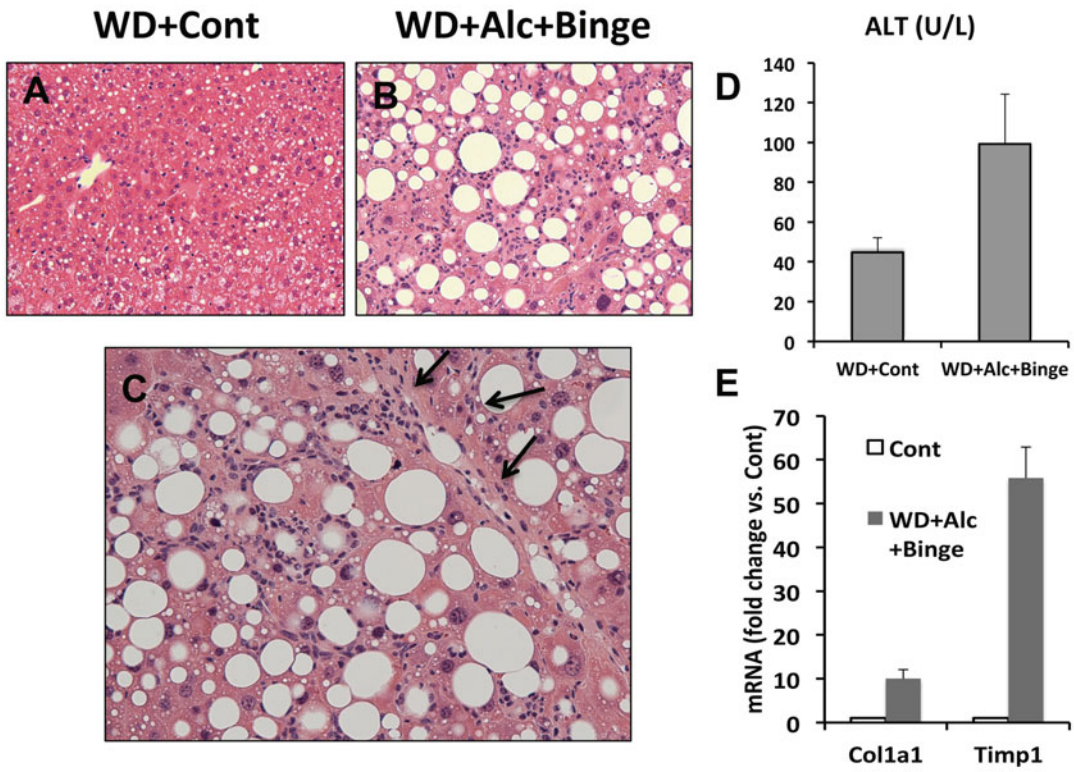

Fig. 5.4 Western ethanol diet ad lib plus weekly binge. (**a**) A low-power view of H&E-stained liver section of a mouse given Western control liquid diet (0.23 % cholesterol, 20.9 % Cal lard) plus isocaloric weekly dextrose binge for 8 weeks (WD + Cont), showing mild fatty liver. (**b**) A low-power view of H&E-stained liver section of a mouse given Western ethanol liquid diet plus weekly ethanol gavage (3.5–4.5 g/kg) for 8 weeks (WD + Alc + binge), showing alcoholic steatohepatitis. (**c**) A high-power view depicts both mononuclear and neutrophilic infiltration, as well as fibrosis (shown by *arrows*). (**d**) Plasma ALT levels are modestly increased in WD + Alc + binge mice. (**e**) The same regimens were subjected to mice expressing GFP under the promoter of Col1a1 and hepatic stellate cells were isolated by FACS based on UV-fluorescence and GFP. Note that mRNA expression of *Col1a1* and *Timp1*, the bona fide fibrogenic genes, is markedly upregulated in the cells isolated from the WD + Alc + binge mice, supporting active fibrogenesis in the model

different versions of the mouse iG model which incorporates different 2nd and multiple hits achieving different spectra of ALD pathology as summarized in Table 5.3.

Standard iG The basic model is standard iG model which is based on iG feeding of high-fat liquid diet (~40 % Cal provided by corn oil) with an increasing dose of ethanol (~33 g/kg/day) for 1~8 weeks, achieving the average BACs of 240–320 mg% and plasma ALT of 200–280 U/L. Mild ASH is induced after 4 weeks of feeding. Focal mononuclear cell infiltration is seen but no neutrophilic inflammation and liver fibrosis are evident. Within 3 weeks of feeding, however, bacterial translocation and enteral dysbiosis occur contributing to liver injury [90]. Supplementation with long-chain saturated fatty acids, which are metabolized by commensal *Lactobacillus* and promote their growth, restores eubiosis and intestinal barrier and reduces alcoholic liver injury [91]. Low-fat (8 % Cal) or extra-high-fat (60 %Cal) diet can also be used in this standard iG model. In addition to the dietary modification, the standard iG model may be combined with single injection of iron dextran which selectively increases iron content in macrophages and exacerbates ASH and liver fibrosis [77].

Table 5.3 Different version of mouse iG models

Model	Standard *iG*	Obesity *iG*	WD hybrid	WD hybrid + binge
Regimen	HFD[a]/ethanol iG	170 % Cal HFD + ethanol iG	HCFD[b] ad lib + ethanol iG	WD hybrid + weekly binge
Duration	1–8 weeks	6–8 weeks	10–12 weeks	10–12 weeks
Ethanol dose	~32 g/kg/day	~32 g/kg/day	~27 g/kg/day	~27 g/kg/day 3.5 ~ 5 g/kg binge
BAC[c] (mg/dL)	309 ± 51	285 ± 58	394 ± 89	263 ± 39.1
ALT[c] (U/L)	269 ± 29	392 ± 28	398 ± 38	270 ± 35
Histology	Fatty liver-mild ASH	Severe ASH	Severe ASH with liver fibrosis	Alcoholic neutrophilic hepatitis
Survival[c] (%)	95 %	91 %	81 %	75 %

[a]HFD: high-fat liquid diet containing 40 %Cal corn oil
[b]HCFD: high-cholesterol high-saturated-fat solid diet (WD: Western diet)
[c]Cumulative data from 2012 to 2013

LPS can be enterally administered to reproduce the condition of severe bacterial overgrowth and dysbiosis to achieve similar exacerbation in the standard iG model [76].

Obesity iG Obesity and ethanol consumption are known to synergistically cause advanced liver disease in patients [92, 93]. This synergism is reproduced by the iG model through overfeeding mice with 170 % of daily caloric intake with high-fat liquid diet for 2 weeks to achieve moderate obesity and combining it with ethanol iG infusion for 4–6 weeks [47]. Severe ASH ensues with intense M1 macrophage activation, nitrosative stress, ER and mitochondrial stress, and hepatic adiponectin resistance. Most recently, this model was used to demonstrate the importance of Notch1 pathway in M1 macrophage activation. In this pathway, Notch1 activation reprograms mitochondrial metabolism to support M1 gene activation and is essential for blood monocytes to migrate into ASH livers and to differentiate to M1 macrophages [94].

Western Diet and iG Hybrid Feeding Model The standard iG model lacks physiologic oral feeding behavior. To incorporate this normal feeding activity while maximally controlling ethanol intake by iG, we have generated the hybrid feeding model. In this model, 40 % of daily caloric intake is achieved by ad lib consumption of regular chow or an experimental diet such as Western diet pellets (HCFD: high-cholesterol high-saturated-fat diet; Dyets Inc. #180529), while the remaining 60 % calories are given by iG feeding of high-fat diet plus ethanol or isocaloric dextrose (Fig. 5.5a). Ethanol dose can be gradually increased to 26 g/kg/day, a lower dose which only induces very mild fatty liver and mild elevation of BACs (~89 mg%) and plasma ALT (~60 U/L) in mice given regular chow. HCFD feeding does not cause any liver pathology and does not elevate ALT in iG pair-fed control mice but produces chronic ASH with liver fibrosis and marked elevation of ALT to ~380 U/L in iG ethanol mice (Fig. 5.5b, c). This remarkable synergism achieved by iG feeding of high-fat ethanol diet and ad lib consumption of HCFD, which provides only ~10.5 mg of cholesterol and 20.9 % Cal of saturated fat to a mouse, is accompanied by marked increase in BACs in these mice (Table 5.3). This underscores how the nutritional modification such as HCFD profoundly affects ethanol metabolism and ALD. This ASH with liver fibrosis model was used to demonstrate that approximately 50 % of activated hepatic stellate cells in the model revert to inactivated cells after resolution to normal liver following 7 weeks of abstinence and regular chow feeding [95].

Western Diet Hybrid Feeding Model Plus Binge Epidemiology for alcoholic hepatitis reveals male Hispanics as a high-risk group [96], and HCFD is their common diet. Heavy drinkers who binge drink are also at high risk for alcoholic hepatitis [4, 5, 97]. To reflect these risk behaviors,

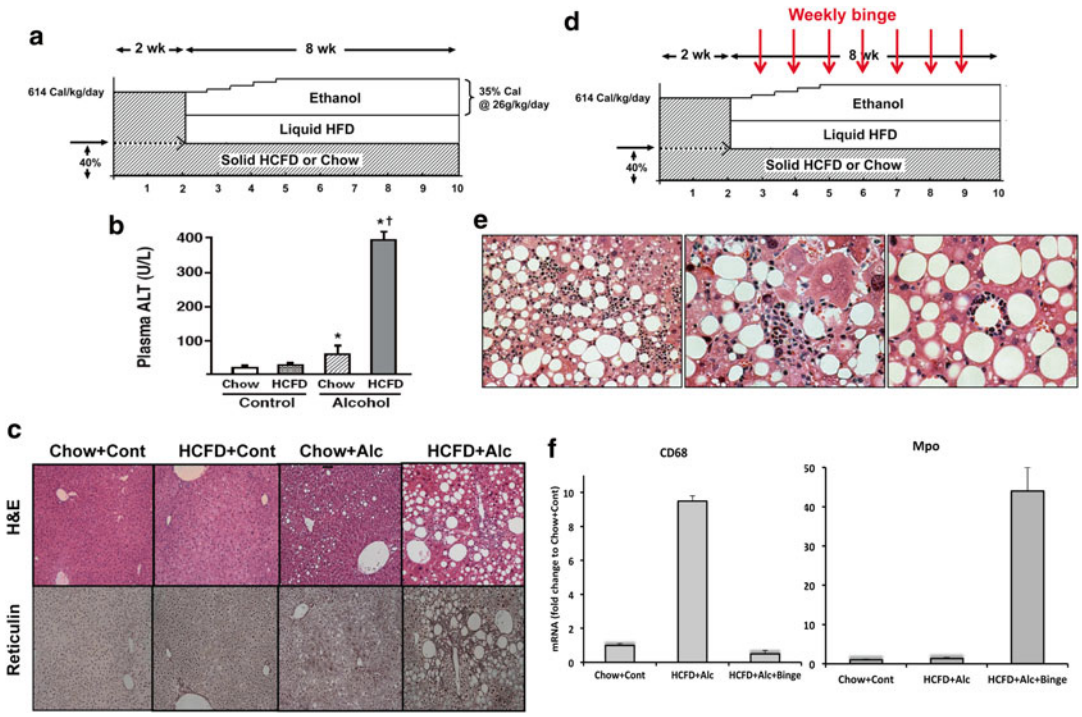

Fig. 5.5 The mouse hybrid feeding models. (**a**) A dietary regimen scheme of the hybrid model. A solid diet high in cholesterol and saturated fat (HCFD) or regular chow is given ad lib for 2 weeks followed by implantation of the *iG* catheter and *iG* feeding of high-fat liquid diet (HFD with 40 % Cal corn oil) and ethanol (gradually increased to 26–27 g/kg/day) accounting for 60 % of daily caloric intake for 8 weeks. The remaining 40 % calories are consumed ad lib from HCFD or chow. (**b**) Plasma ALT levels achieved in the four groups of mice. $*p<0.05$ compared to chow + control; $+p<0.05$ compared to chow + ethanol. (**c**) Representative microphotographs of liver sections stained with H&E and reticulin staining. Note severe fatty liver with mononuclear cell infiltrations and fibrosis in HCFD + ethanol mouse liver. (**d**) The hybrid plus weekly binge model. The regimen is identical to the hybrid model shown in Fig. 5.4a except a bolus of ethanol is given weekly from the 2nd week of *iG* feeding at the dose equivalent to the amount of ethanol withdrawn prior and after the binge (3.5 ~ 5 g/kg/day). (**e**) Low- and high-power views of H&E-stained liver sections with neutrophilic inflammation. (**f**) qPCR results of CD68 and myeloperoxidase (Mpo) mRNA levels in the livers. Note CD68 induction in the hybrid model (HCFD + Alc) which completely disappears in hybrid + binge model (HCFD + Alc + binge). In contrast, Mpo is induced conspicuously in the hybrid + binge model

we have added weekly binge to the Western diet hybrid feeding model with chronic ASH. To implement a binge, ethanol infusion is withdrawn for 4–5 h and the amount withdrawn (4~5 g/kg) is given as a bolus via the iG catheter (Fig. 5.5d). Thus the overall ethanol intake for these models without or with binge is equivalent, but this weekly binge causes a drastic shift of liver pathology from chronic ASH to alcoholic neutrophilic hepatitis (Fig. 5.5e) [55]. Chronic ASH with macrophage activation evident histologically and by upregulation of the macrophage marker CD68 is converted to neutrophilic inflammation with conspicuous myeloperoxidase upregulation and downregulation of CD68 (Fig. 5.5f). Liver fibrosis, as detected by Sirius red staining and fibrogenic gene upregulation (*Col1a1*, *Timp1*, *Acta2*), is more intensified. Further, histological induction of AH is accompanied by some of the known clinical features of AH patients such as splenomegaly indicative of portal hypertension, hypoalbuminemia, and hyperbilirubinemia [55]. Mild abdominal effusion was also noted in some of the mice. Microarray analysis of liver RNA reveals that neutrophil-related genes are markedly upregulated, and qPCR confirms conspicuous induction of *Cxcl1 (Gro)*, *Spp1* (*osteopontin)*, and *Il-17* while *Il-22* is severely downregulated.

TLR4 upregulation and activation are also evident in the AH livers, as well as the appearance of liver progenitors positive for Nanog and α-fetal protein, another feature of AH in patients [98]. As SPP1 is implicated in neutrophil infiltration in the NIAAA model with 10-day L/D diet plus binge [99], *Spp1−/−* mice were also subjected to the Western diet hybrid model with binge (AH model) or without binge (chronic ASH model). The results were surprising but compelling: SPP1 deficiency aggravated AH in the AH model and even promoted AH induction in the chronic ASH model without binge, suggesting SPP1 is protective rather than causal for AH. These discrepant results between the two studies using the two different models underscore the importance of the disease severity or stage in assessing the functionality of one molecule in ALD pathogenesis: the NIAAA model produces mild liver injury while the hybrid plus binge model induces more severe, clinically relevant AH. While SPP1 expression in early liver injury in the former model may be chemoattractive for inflammatory cells, it may be expressed in different cell types in the latter model to provide protective functions. Indeed, in the mouse AH livers, SPP1 expression was detected in multiple cell types including ballooned hepatocytes and hepatic stellate cells [55].

Current Consensus and Future Perspective

Investigators in the ALD field have struggled to provide sufficient ethanol and to achieve advanced ALD spectra in animal models for the past 8 decades. They also faced difficulties in dissociating nutritional effects from ethanol's effects in these models. A comprehensive review of the literature provides us compelling evidence that (1) heavy and steady ethanol intake is required but it alone is not sufficient to produce advanced ALD and (2) recapitulating behaviors of alcoholic patients are important including risk factors such as Western diet and binge. Due to the intense dispute over the nutrition vs. toxicity in the past, the field has been fixated on the idea of maintaining "adequate nutrition" for animal studies on ALD. As the predecessors experienced in 1930–1970 and we have experienced in recent years, nutrition cannot be separated when ethanol is incorporated into feeding. A paradigm shift is required to faithfully reproduce ALD patients' conditions and behaviors in animal models, even including marginal nutrition or malnutrition. Only then clinically relevant models will be developed. Another factor that has influenced the investigators is economy. Tight research funding has placed pressure on us to be inclined toward less expensive and short-duration models. Critical evaluation of the clinical relevance of results generated from different models is an important responsibility we all need to assume.

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Animal Models of Nonalcoholic Fatty Liver Disease

6

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Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
MCD	Methionine-choline deficient
VLDL	Very-low-density lipoprotein
SCD-1	Stearoyl-coenzyme A desaturase-1
PEMT	Phosphatidylethanolamine *N*-methyltransferase
SAMe	S-adenosyl methionine
MAT	Methionine adenosyltransferase
GSH	Glutathione
ROS	Reactive oxygen species
MTHFR	Methylenetetrahydrofolate reductase
ALT	Alanine aminotransferase
TGF	Transforming growth factor
LPS	Lipopolysaccharide
HFD	High-fat diet
GTG	Gold thioglucose
oxLDL	Oxidized low-density lipoprotein
ALIOS	American lifestyle-induced obesity syndrome
CLA	Conjugated linoleic acid
CDAA	Choline-deficient L-amino acids defined
DEN	Diethylnitrosamine
JAK	Janus kinase
STAT	Signal transducer and activator of transcription
MAPK	Mitogen-activated protein kinase
PI3K	Phosphoinositide 3-kinase
AMPK	AMP-activated protein kinase
MC-R	Melanocortin receptor
NOD	Nonobese diabetic
OLETF	Otsuka Long-Evans Tokushima fatty
CCK	Cholecystokinin
SREBP	Sterol regulatory element-binding protein
PEPCK	Phosphoenolpyruvate carboxykinase
AOX	Acyl-coenzyme A oxidase
PPAR	Peroxisome proliferator-activated receptor
MTP	Microsomal trifunctional protein
PTEN	Phosphate and tensin homologue deleted on chromosome 10
KO	Knockout
CBS	Cystathionine β-synthase
IL	Interleukin
IKK	IkB kinase
NFkB	Nuclear factor kappa β

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is the pandemic chronic liver disease of the Western world [1]. Epidemiologic studies suggest that at least 25 % of adults in the United States have some form of NAFLD [2]. NAFLD is characterized by the accumulation of triglyceride in hepatocytes (hepatic steatosis). It is estimated that about a quarter of the NAFLD population (i.e., about 6 % of the general US adult population) has nonalcoholic steatohepatitis (NASH) [3], a more significant form of fatty liver damage with increased hepatic inflammation and hepatocyte injury. In a subset of those with NASH, regeneration cannot keep pace with hepatocyte death, and scarring (fibrosis) ensues. When fibrosis is progressive, cirrhosis eventually results. This dramatically increases the risk for liver-related morbidity and mortality. Although only about 10 % of individuals with NAFLD develop cirrhosis, NAFLD has become a major cause of cirrhosis in the United States because it is the country's most prevalent chronic liver disease. Currently, about 2 % of American adults are thought to have NAFLD-related cirrhosis. Although older age, obesity, diabetes, and other comorbidities often preclude liver transplantation in NAFLD patients, NAFLD-related cirrhosis is already the third most common indication for liver transplantation in the United States [4]. Similar trends are occurring in other countries. Thus, it is crucial to better understand the pathogenesis of NAFLD, to differentiate which patients are most susceptible to progressive liver disease, and to develop better therapeutic tools.

Human investigation in NAFLD, though necessary to obtain a better understanding of this disease, is hampered by several limitations. First, cirrhosis and its complications evolve slowly in NAFLD, as in many chronic liver diseases, necessitating lengthy studies to determine how interventions impact the natural history of the disease. Second, NAFLD encompasses a heterogeneous group of liver pathologies (i.e., diverse liver phenotypes) that are accurately classified only by liver biopsy, an invasive procedure that prompts ethical concerns when applied solely for research purposes. Ethical constraints also govern intervention studies, limiting efforts to clarify cause-effect relationships between NAFLD correlates and disease outcomes. Given these issues, animal models that try to mimic various NAFLD pathologies have been highly used.

There are numerous animal models for NAFLD, which reflects the heterogeneity of NAFLD phenotypes, as well as the fact that the perfect model for fibrosing NASH has yet to be found. The perfect animal model for this ominous NAFLD phenotype would exhibit all histological features of NASH (i.e., steatosis, lobular inflammation, and hepatocellular ballooning), gradually develop perisinusoidal and pericellular fibrosis that advances to cirrhosis, and be prone to hepatocarcinogenesis. Its liver phenotype would occur in the context of obesity, insulin resistance, and the metabolic syndrome and its associated altered adipokine profile. In addition, the ideal model would require relatively little time and cost to maintain, and it would be highly reproducible. To date, rodents, particularly mice, have been the preferred animal models for NAFLD, because they are easy to breed and maintain in an animal facility. Also, genetic manipulations are readily performed in mice, and there is a high genetic similarity between mice and humans. On the other hand, metabolic rates are clearly different between rodents and humans, and they also have distinctive immune systems [5]. The currently available animal models of NAFLD can be subdivided into dietary models, genetic models, and combined dietary-genetic models. We will critically review the available animal models, pointing out their respective advantages and limitations.

Dietary Animal Models

Methionine-Choline-Deficient Diet Model

Feeding rodents a methionine-choline-deficient (MCD) diet is one of the most used models of NASH. The MCD diet is high in sucrose (40 %) and fat (10–20 %) but lacks methionine and

choline. This diet leads to hepatic steatosis because it impairs fat export from the liver as very-low-density lipoproteins (VLDL), compromises hepatic β-oxidation of fatty acids, and increases hepatic uptake of fatty acids [6, 7]. In addition to inducing hepatic fat accumulation, the MCD diet changes the hepatic lipid profile, increasing the saturated versus monosaturated fatty acid ratio and triggering a disproportionate accumulation of polyunsaturated fatty acids. The altered lipid profile may be explained by the downregulation of stearoyl-coenzyme A desaturase-1 (SCD-1), which catalyzes the conversion of saturated fatty acids (e.g., palmitate and stearate) into their monosaturated derivatives (e.g., palmitoleate and oleate) [8]. The MCD diet also restricts the synthesis of phosphatidylcholine, which is an essential component of VLDL, as well as a major biliary phospholipid that catalyzes biliary micelle formation. Phosphatidylcholine can be synthesized through 2 pathways: incorporation of choline into phosphatidyl compounds or through three sequential methylations of phosphatidylethanolamine by phosphatidylethanolamine *N*-methyltransferase (PEMT), using *S*-adenosyl methionine (SAMe) as a methyl donor [6]. SAMe is produced from methionine and ATP, through reactions catalyzed by methionine adenosyltransferases (MAT) [9]. By depriving the animals of both methionine and choline, the MCD diet hampers both pathways that synthesize phosphatidylcholine. Distinct liver phenotypes are observed when one or the other amino acid is selectively depleted, however. Whereas rats fed the MCD diet develop NASH (i.e., hepatic steatosis, inflammation, and liver cell death), adding back methionine prevents NASH, although simple steatosis persists [10]. Methionine-mediated rescue from liver inflammation and injury may reflect the fact that methionine and SAMe-dependent methylation reactions are important for glutathione (GSH) synthesis, protection from reactive oxygen species (ROS) injury, DNA methylation, membrane fluidity, and equilibrium between proliferation and apoptosis [11]. Caballero et al. fed mice with either a methionine-deficient diet or a choline-deficient diet and found that the former was associated with more severe hepatocellular injury, oxidative stress, inflammation, and fibrosis, whereas the latter caused more severe steatosis [12]. The high-sucrose content of the MCD diet accentuates its ability to induce steatosis and liver injury, by providing a lipogenic substrate [8, 13].

Mice fed the MCD diet reproducibly develop steatosis and steatohepatitis within 2 weeks. Steatosis progressively worsens, being predominantly macrovesicular and affecting mainly portal areas (whereas macrovesicular steatosis predominately localizes around terminal hepatic venules in human NAFLD). Hepatocellular ballooning is not prominent in this model (also unlike human NASH). Like human NASH, however, liver cell death (caused by necrosis and lipoapoptosis) and inflammation are exuberant in MCD diet-fed mice, which develop many hepatic necroinflammatory foci, containing lymphocytes, neutrophils, and activated Kupffer cells [14, 15]. Perisinusoidal and pericellular fibrosis emerges within 4 weeks, is reproducibly robust at week 10, and is maximal by week 16 [16, 17]. Interestingly, MCD diet-induced liver fibrosis is reversible if standard chow diets are resumed within 16 weeks of MCD diet initiation, but it cannot be reversed if chow feeding is resumed after that time point [16, 18]. These findings suggest that MCD diet-induced fibrosis is only somewhat reversible, similar to fibrosis in human NASH. Also, as noted in NASH patients who develop advanced liver fibrosis, steatohepatitis (particularly steatosis) lessens in mice with advanced liver fibrosis due to prolonged MCD diet feeding [16].

Gender influences NAFLD susceptibility in humans. Gender differences have also been reported in rodents fed methionine- or choline-deficient diets. As noted in human NAFLD, female rodents are protected from steatosis when compared to male rodents fed choline-deficient diets. This protection is believed to reflect the fact that females have a greater propensity to synthetize phosphatidylcholine via the SAMe-dependent pathway when dietary choline is restricted [19]. Females are also protected from MCD diet-induced steatosis. This is thought to

result from the female's superior ability to regenerate methionine after consumption of SAMe in methylation reactions. In methylation reactions, SAMe donates its methyl groups and is converted into S-adenosylhomocysteine. After hydrolysis, the resultant homocysteine can be methylated to regenerate methionine by methylenetetrahydrofolate reductase (MTHFR) [6]. Ethnicity significantly influences NAFLD susceptibility in humans. Similarly, the severity of hepatic injury induced by the MCD diet is highly dependent on the species and strain of rodent. Mice generally develop worse hepatic necroinflammation than rats. Among rats, Wistar rats seem most susceptible to liver injury [20]. Rangnekar evaluated the effects of MCD diet administered for either 4 or 10 weeks to 8–10 weeks old mice from seven different inbred strains: A/J, AKR/J, BALB/cJ, C57BL/6J, DBA/2J, C3H/HeJ, and 129x1/SvJwt, describing important differences in susceptibility to diet-induced aminotransferase elevation, liver weight gain, and liver fibrosis. Serum aminotransferase levels were higher in A/J, followed by C57BL/6J, whereas liver injury and susceptibility to hepatocarcinogenesis with long-term diet was higher in DBA/2J and C57BL/6J. The authors also found significant mortality in C3H/HeJ, BALB/cJ, and 129x1/SvJwt with long-term feeding [21]. Some of the inconsistencies between diet-induced aminotransferase elevations and diet-induced liver injury might reflect the limitations of currently available noninvasive markers for liver injury. Indeed, it was recently shown that MCD diet-induced increases in alanine aminotransferase (ALT) reflect changes in metabolism, making serum ALT an imperfect surrogate for liver cell injury. Liu et al. fed A/J female mice with MCD diet for 12 weeks and found minimal hepatic inflammation, necrosis, or apoptosis, despite documenting a fourfold increase in serum ALT. The increased serum ALT correlated with increased hepatic expression of the ALT1 and ALT2 genes, consistent with the fact that ALT is an enzyme involved in glycolysis and gluconeogenesis, catalyzing the conversion of L-alanine and α-ketoglutarate into L-glutamate and pyruvate during intermediary metabolism [22]. Increases in alkaline phosphatase of at least 50 % have also been documented in MCD diet-fed rodents, suggesting bile duct injury may occur [23]. This might reflect diet-related depletion of biliary phosphatidylcholine, given that the latter has been implicated in biliary injury in mdr2-deficient mice [24]. Whether or not polymorphisms of genes involved in intermediary metabolism or phosphatidylcholine trafficking contribute to heterogeneity in human NAFLD phenotypes has not been reported. On the other hand, it is well accepted that portal hypertension portends a poor prognosis in NAFLD patients. Recent studies have demonstrated that MCD diet-fed mice develop portal hypertension. This begins before fibrosis is evident and is associated with increased mesenteric arterial and portal venous flow, as well as arterial hyporesponsiveness to vasoconstrictors. Increased intrahepatic resistance from mechanical factors (e.g., sinusoidal narrowing by steatotic and swollen hepatocytes) and adipocytokine effects on contractile function of hepatic stellate cells and sinusoidal endothelial cell biology were also speculated as being contributing factors [25, 26].

Despite its many similarities with fibrosing NASH in humans, the MCD diet model has been criticized because it lacks certain metabolic risk factors that seem important in human NAFLD. Mice fed the MCD diet are not obese. Rather, they develop significant weight loss [27]. NAFLD in humans is strongly associated with obesity. On the other hand, adiposity does not strictly correlate with the severity of NAFLD-related liver injury or fibrosis in humans. Many morbidly obese humans with NAFLD have neither NASH nor liver fibrosis [28], while both liver phenotypes occur in lipodystrophic patients. Weight loss in MCD diet-fed rodents seems most related to choline deficiency, since it is only partially prevented by adding methionine, but it is likely to be multifactorial [10]. Choline deficiency and, to a lesser extent, methionine deficiency decrease intestinal absorption of dietary fat, leading to steatorrhea and reduced capture of dietary energy [29]. MCD diets also induce hypermetabolism. In part, this results from an increase in sympathetic nervous system outflow to adipose tissue during chronic methionine

depletion [30]. MCD diets increase lipase activity in visceral adipose tissue, permitting an increased flux of fatty acids into liver mitochondria. This increases mitochondrial uncoupling, which decreases the efficiency of energy extraction from nutrients and reduces hepatic ATP synthesis [8]. Diet-related suppression of SCD-1 further enhances fatty acid oxidation and energy expenditure [31]. Consistent with the concept that MCD diet feeding causes hypermetabolism, mice fed MCD diet for 3 weeks expended 37 % more energy than chow-fed controls. Moreover, the MCD diet-fed group did not increase food consumption, although they drank fourfold more water than controls [30, 32].

The adipokine profile of MCD diet-fed rodents is also unlike that of patients with NASH. NASH patients generally exhibit hyperleptinemia and reduced circulating levels of adiponectin, while mice with MCD diet-induced NASH have low levels of leptin and no decrease in adiponectin [6]. It is worth noting, however, that hyperleptinemia in humans is often accompanied by some degree of leptin resistance. Like low leptin levels, leptin resistance reduces leptin signaling in relevant target tissues. Thus, leptin activity is variably inhibited in both human NASH and rodents with MCD diet-induced NASH. On the other hand, humans with NASH appear to be relatively deficient in adiponectin, whereas at least the expression of this adipocytokine is maintained in the MCD diet-fed model.

The lipid profile of MCD diet-fed rodents is also different from NAFLD patients, with the model demonstrating decreased levels of total cholesterol and triglycerides relative to the patients [33]. The significance of this disparity is uncertain however, since there is no evidence that NAFLD severity in humans correlates with serum levels of either lipid. Finally, mice fed MCD diet have decreased serum levels of insulin and glucose and demonstrate increased peripheral insulin sensitivity. Hyperinsulinemia and systemic insulin resistance are hallmarks of human NASH [34]. However, the role of insulin resistance in NASH pathogenesis is unclear. Mice with liver-targeted overexpression of PI3K or knockout of PTEN are exquisitely sensitive to insulin and demonstrate robust hepatic insulin signaling. Yet, they develop steatosis, steatohepatitis, liver fibrosis, and liver cancer [35]. Moreover, discordance between improvements in insulin resistance and improvements in liver histology were recently documented in at least two large human NASH treatment trials [36, 37]. The issue is further confounded by a report that MCD diet-fed rodents exhibit features of hepatic insulin resistance despite having enhanced peripheral insulin sensitivity [38, 39].

Several refinements to the diet have been made. Increasing cholesterol content of the diet (1 % by weight) increased liver fibrosis correlating to an increase in free cholesterol in hepatic stellate cells and its sensitization to transforming growth factor (TGF)-β-induced activation [40]. Also, administration of repetitive low doses of lipopolysaccharide (LPS) in mice fed MCD diet increased hepatic inflammation and apoptosis, oxidative stress, and fibrosis [41, 42]. Finally, feeding rats previously loaded with iron with MCD diet during 4 weeks increased necroinflammation, with a trend toward increased perisinusoidal fibrosis [43]. Adding the methionine adenosyl transferase 1 inhibitor, ethionine, to the drinking water further increases MCD diet-related liver damage: mice fed MCD diets with ethionine (MCDE diets) develop NASH more rapidly than those fed MCD diets alone. Because ethionine also inhibits hepatocyte proliferation, MCDE diets mobilize liver progenitors and, thus, are a good model for studying the progenitor response to liver injury [44].

High-Fat Diet Model

Regular High-Fat Diet

Dietary fat requirements in rodents are different from humans. Human diets are considered to be high fat when fat comprises more than 30 % of total energy requirements [45]. However, fat contributes only 5 % of total energy requirements in normal rodent chow [46]. Hence, healthy rodent diets typically contain sixfold less fat than healthy human diets. That said, dietary fat content has varied widely in published studies

of high-fat diet in rodents, ranging from 20 % to more than 70 % of total energy requirements. This variability makes it difficult to compare study outcomes. The issue is further confounded by the fact that the studies often differ with regard to other key variables that may influence liver outcome, such as species, strain, and gender of the rodent used, dietary fat composition and duration of diet exposure, and age of the rodents when high-fat diets were introduced. The latter variable seems to be particularly important, as worse liver damage inevitably occurs when a given diet is started immediately post-weaning, rather than later in life.

Studies using high-fat diet (HFD) to induce NAFLD have mainly been done in rats and mice. For example, male Sprague-Dawley rats were fed a liquid, Lieber-DeCarli diet in which fat contributed 71 % energy ad libitum for 3 weeks [47]. As compared to low-fat isocaloric diet-fed rats, the HFD group did not become obese but developed insulin resistance and liver pathology with panlobular steatosis, mild inflammatory infiltrates, mitochondrial abnormalities, and oxidative stress. There was, however, no increase in the serum aminotransferase levels, and the only evidence of liver fibrosis was an increase in procollagen-1α gene expression [47]. To overcome a potential confounder of ad libitum diet consumption (i.e., self-restriction of caloric intake), the same strain of rats was fed a high-fat diet emulsion via gavage for 6 weeks [48]. When administered HFD via gavage, rats became obese and developed the metabolic syndrome, but liver injury was no worse than when these diets were consumed ad libitum. Lengthening the period of HFD exposure has little impact on liver fibrosis. Only mild fibrosis developed in less than 40 % of rats that were fed HFD for 43 weeks [49]. Strain-dependent differences in HFD outcomes have been reported. Wistar rats are much less sensitive to hepatic steatosis and NASH than Sprague-Dawley rats, even after long-term feeding with HFD [50]. Strain-dependent differences also affect susceptibility to HFD-related liver damage in mice. For example, compared to C57BL/6J mice, BALB/cA mice are more susceptible to HFD-induced steatosis but develop less necroinflammation [51, 52]. Conversely, DBA/2J mice develop less peripheral insulin resistance and milder degree of steatosis than C57BL/6J mice when fed HFD [53]. S129/SVJ and C57BL/6J mice develop similar liver injury after 6 months of HFD. However, the former developed more oxidative stress and demonstrated greater induction of pro-fibrogenic cytokines, such as interleukin 4 and TGF-β [54]. Lastly, unlike A/J mice, which are resistant to weight gain and NASH even when challenged with HFD for 60 weeks, C57BL/6J mice fed HFD for the same time period develop severe NASH and hepatocellular carcinoma [55].

In general, however, mice (like rats) are relatively resistant to HFD-induced liver injury. Most strains develop only mild hepatic inflammation and almost no liver fibrosis unless diet exposure is quite prolonged. For example, although C57BL/6J mice that were fed HFD (with 60 % calories from fat) developed obesity, insulin resistance, and dyslipidemia and mild inflammation, serum levels of aminotransferase elevations and slight liver perivenular fibrosis emerged only after 50 weeks of HFD exposure [56]. The model improves when HFD is administered via implanted gastrostomy tube (allowing overfeeding) but even then, mice develop only modest liver damage. Deng et al. fed mice with a HFD (37 % calories from fat) for 9 weeks, overfeeding in a stepwise manner (130 % of regular calories on day 3, 150 % on day 5, 170 % on day 17, and 185 % from day 19 through 9 weeks) [57]. Body weight increased by 70 %, and insulin resistance and hyperleptinemia developed. However, NASH occurred in less than 50 % of the mice, and perisinusoidal fibrosis was mild [57]. Other investigators [58] obtained similar results. Another interesting approach to increase the food consumption by animals was recently described by Ogasawara et al. [59]. The authors injected mice C57BL/6 with gold thioglucose (GTG) IP (2 mg/g weight) and fed HFD (82 % calories from fat) for 12 weeks, starting immediately after weaning (week 4). GTG induces lesions in the ventromedial hypothalamus leading to hyperphagia and obesity. GTG-treated mice did become hyperphagic and developed obesity with

increased abdominal adiposity, insulin resistance, and adipokine deregulation. All the histological characteristics of NASH, including hepatocellular ballooning and Mallory-Denk bodies, as well as, pericellular fibrosis, also emerged [59].

STAM mice provide another model of accelerated NASH. In this model, male mice are injected with 200 μg of streptozotocin at 2 days of age and fed a HFD (32 % calories from fat, of which 22.3 % are saturated fats) from 4 weeks of age (i.e., about the time of weaning). At 8 weeks, animals develop NASH (with severe hepatocellular ballooning but mild steatosis and inflammation), significant fibrosis emerges 1 week later, and hepatocellular carcinomas are evident after 16 weeks of high-fat diet exposure (20 weeks of age) [60]. Besides the fact that the formal STAM protocol remains unpublished, the model has an important limitation with regard to mimicking the metabolic milieu of human NAFLD, namely, it causes type 1 diabetes (hyperglycemia due to insulin insufficiency), rather than type 2 diabetes (hyperglycemia due to insulin resistance) because streptozotocin destroys pancreatic β cells.

Lastly, it has been suggested that supplementing a regular HFD with IV injections of oxidized low-density lipoproteins (oxLDL) in the last 2 weeks of 23 weeks of HFD feeding might be useful to induce NASH. It was reported that this approach promoted liver injury in C57BL/6 mice, aggravating lipid metabolism, hepatic steatosis, apoptosis, inflammation (with foamy macrophages), and fibrosis [61]. In vitro studies showed that oxLDL could promote hepatic stellate cell activation [62, 63].

Western or Fast-Food Diet

Although standard HFDs do not seem very promising as an approach to induce NASH in rodents, epidemiologic studies in humans suggest that the fat composition of the diet might play a role in NAFLD pathogenesis/progression because the intake of saturated fatty acids and cholesterol positively associates with NAFLD and NASH [64–66]. In humans, high consumption of simple carbohydrates, particularly fructose, also associates with the metabolic syndrome and risk for developing NAFLD, NASH, and advanced fibrosis [67, 68]. Researchers have exploited these insights to create models that mimic this "Western-type" high-fat diet (also known as the "fast-food" diet) by feeding higher amounts of saturated fats and trans fats and increasing dietary cholesterol content. In some circumstances, simple carbohydrates (equivalent to high-fructose corn syrup) are also supplemented to reproduce high-sweetened soda beverage consumption that is typical of humans who habitually ingest "fast-food"-enriched diets.

The American lifestyle-induced obesity syndrome (ALIOS) diet has become a popular Western (fast-food) diet model for inducing NAFLD/NASH. In the ALIOS diet model, 45 % dietary calories are derived from fat (enriched with trans fats from partially hydrogenated vegetable oil), and the high-fat diet is supplemented with sucrose and fructose in the drinking water (42 g/L) [69]. When C57BL/6 mice (aged 5–6 weeks old at the time of diet initiation) were fed ALIOS diets for a total of 16 weeks, they became obese and insulin resistant. Plasma levels of insulin, resistin, leptin, and ALT increased, and there was a progressive increase in hepatic steatosis, hepatocellular ballooning, Mallory-Denk bodies, and necroinflammatory changes. However, despite having NASH and increased procollagen-1α gene expression, mice failed to develop evidence of liver fibrosis, as assessed by trichrome Masson staining, Sirius red staining, or immunohistochemistry for α-smooth muscle actin [69]. Subsequent analyses suggested that outcomes might have been caused by unique components of the ALIOS protocol. Obesity, insulin resistance, and hypertriglyceridemia were attributed to ingestion of the high-fructose corn syrup equivalents that promoted food consumption, whereas hepatic steatosis and injury were attributed to the trans-fat exposure. The latter assumption was challenged by another experiment, which added fructose and sucrose to drinking water of mice that were fed regular high-fat diet (58 % calories as fat, but no trans-fat enrichment), and achieved mild liver fibrosis in 50 % of mice after 16 weeks [70]. Dietary cholesterol content has also been implicated in modulating the propensity for liver fibrosis development during

the ALIOS protocol. Fibrosing NASH and cirrhosis were reported to occur in mice that were fed a diet containing 40 % fat (18 % trans fats), enriched with fructose (22 % by weight) and cholesterol (2 % by weight) for 30 weeks. Although the frequency of fibrosing NASH and cirrhosis was not specified, there was a threefold increase in hepatic hydroxyproline content and collagen-1α expression overall [71]. Another study that used a similar diet achieved comparable results, with stage 2 fibrosis occurring in 6 of 7 mice after 25 weeks [72]. It is important to emphasize, however, that enriching a typical rodent diet with 2 % cholesterol is equivalent to enriching a normal human diet with 2000 mg cholesterol per day. This level of cholesterol ingestion is almost 10 times more than the average daily cholesterol intake [73]. In a more physiological way, similar diets containing as little as 0.2 % cholesterol content can evoke a metabolic profile reminiscent of human NAFLD (obesity, hypertension, hypertriglyceridemia, hypercholesterolemia, and hypertension), as well as the histological features of NASH (steatosis, necroinflammation, hepatocellular ballooning, and some degree of fibrosis) when diet administration is initiated immediately after weaning and continued for 12–16 weeks [74, 75].

Combined Methionine-Choline-Deficient + High-Fat Diet

Cong et al. fed C57BL/6 mice with a modified HFD (60 % calories from fat, of which 30 % saturated fat) for 23 weeks [76]. This diet had low methionine (1.5 g/kg versus 3 g/kg in a regular diet) and choline content (0.6 g/kg versus 2 g/kg in a regular diet). Mice developed the metabolic syndrome (obesity, dyslipidemia, and insulin resistance) and hepatic pathology with severe steatosis, but only scattered foci of lobular inflammation and mild pericellular and perisinusoidal fibrosis developed [76]. More recently, the same strain of mice was fed a 60 % HFD without choline and methionine for 8 weeks. These mice did not become obese (rather, they lost weight and increased peripheral insulin sensitivity), but the diets provoked fibrosing NASH, with prominent lobular inflammation, perivenular/pericellular fibrosis, and a fourfold increase in hepatic hydroxyproline content [77].

Atherogenic Diet

The atherogenic diet, or Paigen diet, contains 1.25 % cholesterol and 0.5 % cholate. When administered to rodents, it induces progressive, time-dependent liver injury, with steatosis, inflammation, important perisinusoidal fibrosis, and hepatocellular ballooning after 6 months. If the diet is also high fat (60 % fat as cocoa butter), it worsens liver fibrosis and accelerates NASH development, with hepatocellular ballooning becoming apparent as early as 3 months [78]. The effect of the diet on the liver is strain dependent, with inflammation and fibrosis being particularly mild in C3H mice [79]. One limitation of atherogenic diets is that they fail to reproduce several of the metabolic abnormalities that are typical in human NAFLD. Even when the Paigen diet is high in fat, it promotes weight loss and decreased visceral adiposity, does not evoke hypertriglyceridemia, and only modestly decreases insulin sensitivity after very long feeding periods [78].

Diet Containing Conjugated Linoleic Acid (CLA Diet)

Conjugated linoleic acid (CLA) is a trans-fatty acid [i.e., a mixture of positional and geometric isomers of linoleic acid (18:2, n6)]. One such isomer, trans-10, cis-2 CLA, is found in partially hydrogenated vegetable oil and in processed foods. Feeding 8-week-old female C57BL/6 mice with a diet containing 0.5 % CLA for 8 weeks induced insulin resistance and decreased both leptin and adiponectin. Hepatic macrosteatosis occurred as a result of diet-enhanced lipogenesis and decreased β-oxidation of fatty acids. Hepatocellular apoptosis, inflammatory infiltrates, and increased number of Kupffer cells as well as hepatic stellate cell activation and mild perisinusoidal fibrosis were also noted [80–82].

Choline-Deficient L-Amino Acid-Defined Diets

The choline-deficient L-amino acid-defined (CDAA) diet consists of a diet with low choline content and in which amino acids are all pure L-enantiomers. When given to Fisher rats for 10 weeks, CDAA diets induce fibrosing NASH, with high levels of oxidative stress [83]. At 12 weeks some rats exhibit cirrhosis [84]. If administered for 1 year, 100 % animals developed hepatocellular carcinoma [84]. However, this diet induces weight loss (though with increased visceral adiposity), increased levels of adiponectin, and less insulin resistance, unlike human NASH [83]. The mechanism by which CDAA diets exacerbate fatty liver damage is not well understood. However, it has been suggested that the lack of oligopeptides may decrease the absorption of methyl donor amino acids and antioxidant minerals [84]. The combination of CDAA with HFD (35 % calories as fat), enriched with trans-fatty acids (54 %) and exposure to diethylnitrosamine (DEN) in drinking water, accelerates liver pathology, with almost all rats developing frank cirrhosis and hepatocellular carcinoma in 16 weeks [85].

CDAA and HFD administered to mice induce similar lesions, though not so severe [86].

Summary

MCD diet-based NAFLD models generally induce more severe NASH-like lesions, including intense necroinflammation and fibrosis, than HFD-based NAFLD models. Compared to HFD models, MCD diet models are also more reproducible and require shorter durations of feeding to induce fibrosing NASH and hepatocellular carcinoma. As such, researchers have extensively used the MCD diet model. However, the MCD model has been criticized because it causes severe weight loss and increased peripheral insulin sensitivity. As such, this model promotes a systemic metabolic phenotype that is very different than most humans with NASH.

HFD-based models have the advantage of mimicking the metabolic background that occurs in human NAFLD (i.e., obesity, insulin resistance, and dyslipidemia), although several diets do not achieve hypertriglyceridemia. The disadvantage of the traditional HFD model is that it rarely induces NASH or fibrosis. Generally, very long periods of diet administration are required to evoke even a low frequency of these important liver outcomes. More recently, investigators are changing the composition of high-fat diet models to mirror a Western fast-food diet, with more saturated and trans fats, high cholesterol content, and supplementation with high-fructose corn syrup equivalents. These changes in diet seem to enhance liver injury, with fibrosis stage 1–2 appearing after 12–16 weeks. The lesions, however, are still generally less severe than observed with the MCD model, and not all HFD animals achieve the desired hepatic phenotype of fibrosing NASH (Table 6.1).

Genetic Models (Table 6.2)

Rodents with Disturbed Appetite Regulation

ob/ob Mice

The ob/ob phenotype resulted from a spontaneous mutation in an outbred colony at Roscoe B. Jackson Memorial Laboratory in 1949 and subsequent transfer of the mutation onto a C57BL/6 background [87]. Forty-five years later, Jeff Friedman discovered that the mutated gene responsible for the phenotype was leptin, an adipokine produced mainly in white adipose tissue [88]. The main signaling pathways of leptin are Janus kinase and signal transducer and activator of transcription (JAK-STAT), mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), and AMP-activated protein kinase (AMPK) pathways [89]. Leptin receptor has different isoforms generated by alternative splicing of leptin mRNA [90]. The long form of the receptor (Ob-Rb) encoded by the full-length leptin transcript) is necessary for activation of

Table 6.1 Dietary rodent models of NAFLD/NASH

Model	Description	Advantages	Limitations
Methionine-choline-deficient diet (MCD)	• High-sucrose (40 %) and high-fat (10–20 %) diet lacking methionine and choline • Impaired VLDL assembly and β-oxidation of fatty acids	• Day 10: NASH • 6–10 weeks: perisinusoidal fibrosis • Highly reproducible • Lower diet duration, compared to other diets • Higher fibrosis, inflammation, oxidative stress, and apoptosis than other models	• >30 % body weight loss • ↓ leptin levels and no ↑ adiponectin • Low glycemia, peripheral insulin sensitivity (though with hepatic insulin resistance) • No clear hepatocyte ballooning
High-fat diet	• Usually >60 % calories from fat	• Develops obesity, insulin resistance, and dyslipidemia • Hepatic steatosis	• Effects strain and gender dependent • Variable liver injury even in the same cohort • Very mild inflammation • Fibrosis very mild and only after at least 1 year of treatment
Western/fast-food diet	• ↑ % saturated and trans fats • High cholesterol intake HFCS equivalents	• Develop obesity, insulin resistance, and dyslipidemia • NASH with hepatocellular ballooning • Variable degrees of fibrosis	• Longer duration of diet, usually 16 weeks (12–20 weeks) • Not all mice develop fibrosis
Atherogenic diet or Paigen diet	• Diet with 1.25 % cholesterol and 0.5 % cholate	• Fibrosing NASH with hepatocellular ballooning	• It needs 6 months of diet (3 months if high fat also) • Weight loss and ↓ visceral adiposity • No hypertriglyceridemia or insulin resistance
CLA diet	Supplementation with the trans-fat-conjugated linoleic acid	• Insulin resistance • Steatosis, apoptosis, and inflammation	• Only with mild perisinusoidal fibrosis
CDAA diet	• Diet choline deficient, L-amino acid defined	• Fibrosing NASH and even cirrhosis in rats, after 3 months • Hepatocellular carcinoma in 1 year • Enhanced liver injury if also high-fat diet	• Weight loss • ↑ adiponectin • Insulin sensitivity

MCD methionine-choline-deficient diet, *VLDL* very-low-density lipoproteins, *NASH* nonalcoholic steatohepatitis, *HFCS* high-fructose corn syrup

JAK-STAT pathway. Ob-Rb acts both centrally in the nervous system and in peripheral tissues, such as the adipose tissue, muscle, liver, and pancreatic islets. The short form of the leptin receptor (Ob-Ra), which lacks the cytosolic domain of Ob-Rb, is localized predominantly in the skeletal muscle and activates the PI3K pathway [89]. In the hypothalamus, leptin acts in the ventral median nucleus as a potent anorexic agent [6]. Besides regulating satiety, leptin increases energy expenditure, promotes physical activity, enhances thermogenesis, increases sympathetic tone, and regulates immune cells, such as T cells and macrophages, among many other actions. Consequently, ob/ob mice are

Table 6.2 Genetic rodent models of NAFLD/NASH

Model	Description	Advantages	Limitations
ob/ob	• Spontaneous mutation in leptin • Leptin deficiency leads to disturbed appetite regulation	• Mice are hyperphagic, inactive, obese, with insulin resistance and dyslipidemia • Severe hepatic steatosis • MCD diet leads to features of NASH	• No hypertension • NASH requires a second hit (MCD or HFD) • Resistant to fibrosis, even after a second hit
db/db	• Mutation in leptin receptor (Ob-Rb)	• Obesity and insulin resistance/diabetes mellitus • Macrovesicular steatosis • Fibrosing NASH after MCD diet	• No hypertension • NASH and fibrosis do not occur spontaneously, needs a second hit
aP2-nSREBP-1c transgenic	• Overexpression of SREBP-1c in adipose tissue, under regulation of aP2 promoter • Impaired adipose tissue differentiation	• Hyperglycemia and ↓ adiponectin • Day 8: steatosis • 20 weeks: NASH; mild fibrosis (in 54 %) • 30 weeks: fibrosis in 93 %	• Model of lipodystrophy-associated NASH • Hypoleptinemia
AOX KO	• Deficiency in acyl-coenzyme A oxidase, rate limiting of peroxisomal β-oxidation	• Day 8: steatosis • 2 months: inflammation, apoptosis, regenerating hepatocytes • 8 months: fibrosis with bridging • 15 months: hepatocellular carcinoma	• Fibrosis only after 8 months of age
PTEN null mice	• $PTEN^{loxP/loxP}$; $Alb\text{-}Cre^{+}$ • Liver-specific KO for the tumor suppressor PTEN	• 10 weeks: steatosis • 40 weeks: fibrosing NASH • 74 weeks: hepatocellular carcinoma in 100 %	• No obesity or metabolic syndrome • Insulin hypersensitivity • Phenotype acquired at advanced age
MAT1A KO	• Deficiency in methionine adenosyltransferase leading to impaired antioxidant defense and lipid metabolism	• Spontaneous NASH at 8 months • Higher susceptibility to tumors	• Hyperglycemia but normal insulin • Do not develop metabolic syndrome • Fibrosis not described

MCD methionine-choline deficient, *NASH* nonalcoholic steatohepatitis, *HFD* high-fat diet, *KO* knockout, *PTEN* phosphatase and tensin homologue

hyperphagic, inactive, and markedly obese (up to four times the weight of wild-type animals), have all the features of the metabolic syndrome except hypertension, and exhibit impaired immune function [91]. ob/ob mice are indistinguishable from wild-type mice at birth but start to gain weight and become hyperinsulinemic by 2 weeks of age. Hyperglycemia is evident by 4 weeks of age and steadily worsens until 3–5 months of age. After that, glycemia decreases and eventually normalizes with aging [89].

ob/ob mice have deregulated lipid and bile acid metabolism and develop severe hepatic steatosis on regular chow diets [92]. NASH occurs with addition of secondary insults, such as HFD [93–95], or MCD diet [96, 97], or low dose of LPS [98]. However, ob/ob mice are very resistant to fibrosis, even when other triggers for liver injury, such as chronic carbon tetrachloride treatment, are applied [99, 100], revealing the important role of leptin in liver fibrogenesis [91]. Also, the associations between leptin and the sympathetic nervous system seem important, since treating ob/ob mice with norepinephrine shifts the liver immune microenvironment to a more fibrogenic Th2 response and increases recruitment of NKT cells, promoting LPS toxicity and fibrogenesis [101, 102].

In conclusion, although mutations in ob gene are very rare in obese humans with NAFLD [6], functional leptin deficiency caused by hyperleptinemia-induced leptin resistance is common in human obesity. This may explain why the leptin-deficient, ob/ob mouse is a good model for human NAFLD. Like humans with NAFLD, ob/ob mice have metabolic syndrome-associated steatosis and develop NASH when challenged with secondary metabolic/inflammatory stressors. However, ob/ob mice are more resistant to liver fibrosis than most NAFLD/NASH patients and thus, they are not a useful model for studying the pathogenesis of NASH cirrhosis.

db/db Mice

Mice with db/db phenotype were first described in 1996 as a model of type 2 diabetes [103]. They carry a recessive mutation in the leptin receptor gene (Ob-Rb) on mouse chromosome 4 [104]. The db mutation leads to premature termination of Ob-Rb long intracellular signaling domain, resulting in leptin resistance despite normal or increased leptin levels [104]. db/db mice start to gain weight at 3–4 weeks of age and shortly thereafter develop hyperglycemia, polyuria, and glycosuria. As with ob/ob mice, db/db mice develop all the features of the metabolic syndrome except hypertension. Db/db mice were originally described in the C57BLKS/J strain but were subsequently backcrossed onto C57BL/6J and FVB/NJ strains [105]. The phenotype is more severe in the former.

db/db exhibit liver steatosis, without appreciable hepatic inflammation or fibrosis, when fed normal chow. When placed on MCD diets, db/db mice develop worse and earlier liver inflammation and fibrosis than lean nondiabetic mice [11]. Within 4 weeks, collagen-1α gene expression is 2.5-fold greater in MCD diet-fed db/db mice than MCD diet-fed wild-type controls, and both groups have significantly more fibrosis than MCD diet-fed ob/ob mice [96], demonstrating a role of Ob-Ra (short isoform of leptin receptor) in liver fibrogenesis [106]. HFD also significantly worsens liver injury and fibrosis in db/db mice with one third of HFD-fed db/db mice achieving at least 5 points in NAFLD Activity Score after 3 months of HFD exposure [94]. The aggregate data indicate that the db/db mouse is a good model for human NAFLD. Like most NAFLD patients, db/db mice are hyperleptinemic, are leptin resistant, and have liver steatosis and the metabolic syndrome. In addition, like many NAFLD patients, db/db mice develop NASH when further challenged by additional oxidant/metabolic stress. Finally, progressive liver fibrosis results from NASH in db/db mice, as occurs in some patients with NASH.

fa/fa Zucker Rats

The fa/fa Zucker rat obese phenotype was observed by Zucker et al., in 1961, and demonstrated to result from a spontaneous mutation, named fatty or fa [107]. It is an autosomal recessive mutation that affects the extracellular part of the leptin receptor, leading to weaker affinity for leptin and altered signal transduction [90]. The mutation localizes in +269 codon, replacing a glutamine with a proline, leading to a truncated leptin receptor protein [90].

Zucker fa/fa rats exhibit hyperphagia, obesity, and hyperlipidemia, with plasma triglycerides and cholesterol increasing with age. They exhibit mild insulin resistance and hyperinsulinemia but do not have fasting hyperglycemia. Also, moderate hypertension may develop in older mice. fa/fa rats also develop spontaneous liver steatosis, with liver lipogenesis increasing, but adipose tissue lipogenesis decreasing, with age [108]. However, the hepatic steatosis is mild and does not typically lead to NASH or fibrosis. After consuming high-saturated fat diet (60 % calories from fat as lard) for 8 weeks, fa/fa rats developed overt hyperglycemia and liver injury, with increased levels of ALT, oxidative stress, more steatosis, and mild periportal fibrosis (assessed by Sirius red and hydroxyproline assay) [109]. When given long term with or without low doses of LPS, another diet enriched with disaccharides (12.1 % calories as sucrose or lactose) exacerbated steatosis and induced fibrosis, without worsening liver inflammation. Fibrosis severity increased with diet duration, and bridging was apparent after 24 weeks of diet exposure [110, 111]. Thus, fa/fa rats provide another model of potentially progressive NAFLD that develops in the context of the metabolic syndrome.

Agouti Yellow Mice

Agouti was the first cloned gene for obesity [112]. In wild-type mice, Agouti protein is expressed only in the skin during the neonatal period and in the testis during adulthood. Agouti is normally involved in the regulation of coat color, inducing a subapical yellow band on otherwise-black hair [113]. Agouti mice harbor a spontaneous mutation, Ay, that deletes a fragment of DNA 170 kB upstream of the coding region of the Agouti gene. This DNA deletion removes the Raly (ribonucleoprotein associated with lethal yellow) gene coding sequence, leaving the promoter of the Raly gene (which encodes a ubiquitous protein) to control Agouti gene expression. This causes ectopic expression of Agouti in multiple tissues [90]. Homozygous animals for the Ay mutation do not survive. Heterozygous mice are hyperphagic (though less so than ob/ob mice), obese, insulin resistant, hyperglycemic, dyslipidemic, and hyperleptinemic. They also exhibit increased linear growth and yellow coat color [113].

Agouti functions as a competitive inhibitor for melanocortin receptors (MC-R). MC-R are G-protein-coupled receptors expressed in different tissues, for instance, MC1-R is expressed in melanocytes and regulates pigmentation; MC4-R is present in the hypothalamus and regulates appetite. Agouti prevents the binding of α-melanocyte-stimulating hormone (α-MSH) to melanocortin receptors. This suppresses the production of cAMP. In melanocytes, reduced cAMP shifts synthesis of eumelanin (black pigment) to phaeomelanin (yellow pigment). In the hypothalamus, loss of cAMP inhibits appetite suppression [113]. Leptin promotes the expression of α-MSH and, hence, seems to regulate appetite by acting upstream MC4-R [114]. In the hypothalamus, ectopic Agouti prevents a-MSH from activating MC4-R, thereby blocking the normal actions of leptin on MC4-R signaling. Ectopic expression of Agouti in adipose tissue leads to increased leptin expression and secretion. The resultant hyperleptinemia generally reenforces leptin resistance [115]. However, Agouti mice are not resistant to leptin effects on the sympathetic nervous system and they are hypertensive [90]. On chow diets, Agouti mice develop hepatic steatosis. MCD diets provoke worse NASH and result in more severe fibrosis in Agouti mice than in wild-type mice [116].

MC4-R KO Mice

Mice deficient in MC4-R have a similar phenotype as Agouti mouse, without the yellow discoloration of the coat. In the liver, they develop massive hepatic steatosis associated with increased hepatic lipogenesis [117]. When fed a HFD (60 % calories from fat), MC4-R-deficient mice develop progressive disease with NASH (inflammation, hepatocellular ballooning) and pericellular fibrosis at 20 weeks and hepatocellular carcinoma after one year [118].

Fat Aussie (Alms1 foz/foz) Mice

The Alström syndrome is an autosomal recessive disease characterized by childhood-onset obesity, metabolic syndrome, type 2 diabetes mellitus, and infertility. It is caused by mutations in human ALMS1 gene. Fat aussie (Alms1 foz/foz) is a mouse model of Alström syndrome that results from the spontaneous recessive variant in Alms1 gene in a nonobese diabetic (NOD) mouse colony. The variant consists of a deletion causing a frameshift and premature termination codon [119]. The protein encoded by the wild-type Alms1 gene is expressed in the basal bodies of cilia. Although its biological role is not yet understood, Alms1 seems to be involved in appetite regulation. Alms1 foz/foz mice are normal in weight when young but become hyperphagic at 2 months of age and exhibit obesity, hyperinsulinemia, and diabetes by 4 months of age. Male foz/foz mice are always infertile, but females are fertile until the onset of metabolic disturbances [119]. On chow diet, foz/foz mice develop simple steatosis. When fed HFD, they develop NASH after 3 months and pericellular fibrosis after 6 months. HFD liver injury is significantly worse in HFD-fed foz/foz mice than in wild-type mice that were fed the same diet [120, 121]. Increasing the cholesterol content of the diet progressively worsens liver injury in foz/foz mice [122]. The severity of HFD-induced liver injury is also influenced by genetic background, being more severe in foz/foz C57BL/6 mice than foz/foz BALB/c mice [52].

Otsuka Long-Evans Tokushima Fatty Rats

Otsuka Long-Evans Tokushima fatty (OLETF) rats emerged in an outbred colony of Long-Evans rats at the Tokushima Research Institute in 1984 due to a spontaneous loss-of-function mutation in the cholecystokinin 1 (CCK-1) gene [123]. CCK controls food intake. Hence, loss of CCK-1 function results in hyperphagia and obesity. OLETF rats are frankly diabetic by adulthood, manifesting severe hyperglycemia, polyuria, and polydipsia [90].

OLEFT rats fed standard rodent diet develop NAFLD with steatosis and hepatocellular ballooning at 22–38 weeks. Those features, however, disappear at 42 weeks of age, and there is no fibrosis [124]. When OLEFT rats are fed MCD diet, with or without high-fat content, for 8 weeks, they develop NASH with intense lobular inflammation and perivenular/pericellular fibrosis [125, 126].

Genetic Models with Lipodystrophy-Like Phenotype

SREBP-1c Transgenic Mice

There are two nonobese transgenic mouse models that overexpress sterol regulatory element-binding protein (SREBP), an important transcription factor that regulates lipid and glucose metabolism. In PEPCK-nSREBP-1a transgenic mice, the nSREBP-1a transgene (which encodes a nuclear-targeted SREBP-1a) is under the control of the phosphoenolpyruvate carboxykinase promoter, resulting in deregulated hepatic expression of activated SREBP-1a [127]. These mice develop progressive atrophy of white adipose tissue, increased hepatic lipogenesis and steatosis, and increased ALT [128]. However, spontaneous NASH and dyslipidemia do not occur. In the other transgenic strain, the aP2 promoter controls expression of an nSREBP-1c transgene (which encodes a nuclear-targeted form of SREBP-1c). Because aP2 is an adipose tissue-specific promoter, overexpression of SREBP-1c is localized to adipose depots in these mice. Ap2-nSREBP-1c mice exhibit a severe lipoatrophic phenotype, with very low plasma leptin levels [129]. The animals are hyperphagic, insulin resistant, and hypertriglyceridemic. They develop massive ectopic fat accumulation, including hepatic steatosis, which is evident as early as 8 days of age and worsens with age. By 20 weeks, the mice develop NASH with hepatocellular ballooning and pericellular/perivenular fibrosis [130]. Hence, aP2-nSREBP-1c mice are an excellent model for lipodystrophy-associated NASH.

aP2-Diphteria Toxin Mice

aP2-diphteria toxin mice (aP2/DTA mice) are another model of lipodystrophy-induced NAFLD [131]. In these mice, expression of an attenuated diphtheria toxin A chain has been targeted to adipose tissue, resulting in severe atrophy and necrosis of subcutaneous and intra-abdominal adipose tissue by 8–9 months of age [132].

A-ZIP/F1 Mice

This is also a model of severe lipodystrophy. The mice are hyperphagic, hypoleptinemic (20-fold lower levels than wild-type mice), diabetic, and hypertensive. They develop severe hepatic steatosis [133]. Transgenic mice express a dominant negative protein, A-ZIP/F1, under the control of the aP2 promoter. Hence, transgene expression is targeted to fat. The protein encoded by A-ZIP prevents the DNA binding of B-ZIP transcription factors, such as C/EBP and Jun family members, thereby interfering with adipocyte differentiation. Although this lipodystrophy model results in severe hepatic steatosis, it does not appear to cause spontaneous NASH or fibrosis.

CD36-Deficient Mice

CD36 (fatty acid translocase) is a multispecific transmembrane glycoprotein that acts as facilitator of fatty acid cellular uptake [131]. It is expressed in the liver and peripheral tissue, including muscle and adipose tissue. CD36-deficient mice exhibit impairment in fatty acid storage and hence increased circulating levels of long-chain fatty acids and triglycerides, hepatic insulin resistance (though with muscle insulin sensitivity), and hepatic steatosis [134].

Genetic Models with Primary Altered Lipid Metabolism

Acyl-Coenzyme A Oxidase (AOX)-Deficient Mice

AOX KO mice are deficient in Acyl-coenzyme A oxidase, which is the rate-limiting enzyme of peroxisomal β-oxidation of long-chain fatty acids. These mice exhibit growth retardation and high levels of very long-chain fatty acids. They develop hepatic steatosis as soon as 8 days of age. Over time, hepatic lipid accumulation increases in severity and becomes associated with focal inflammatory cell infiltrate. There is also increased hepatocyte apoptosis and hepatocyte proliferation/regeneration. At the age of 2 months, clusters of regenerating hepatocytes with peroxisome-rich eosinophilic granular cytoplasm (indicative of peroxisomal proliferation) accumulate in periportal areas. Thereafter, steatosis progressively decreases. Starting at 8 months, portal-portal and portal-central bridging fibrosis is evident. At 15 months of age, hepatocellular carcinoma develops. Liver disease pathogenesis is attributed to the accumulation of long-chain fatty acids, leading to oxidative stress and sustained activation of peroxisome proliferator-activated receptor (PPAR)-α. The latter induces endoplasmic reticulum stress, which promotes hepatocyte apoptosis and a regenerative response that increases hepatocarcinogenesis [135, 136].

PPAR-α-Deficient Mice

PPAR-α is a transcription factor that regulates peroxisomal, mitochondrial, and microsomal fatty acid oxidation. PPAR-α is expressed in the liver, adipose tissue, muscle, kidney, and pancreas. Its expression decreases in the liver of rodents submitted to high-fat diet [137]. Mice deficient in PPAR-α develop massive hepatic steatosis during HFD feeding or prolonged fasting (24–72 h). In fact, PPAR-α is crucial to the metabolic adaptation to fasting, and PPAR-a-deficient mice develop severe hypoglycemia, hypoketonemia, and hypothermia in that condition [138].

Microsomal Trifunctional Protein-Deficient Mice

Microsomal trifunctional protein (MTP) is a crucial enzyme for mitochondrial β-oxidation of fatty acids. Homozygous KO mice die 6–36 h post birth due to severe hypoglycemia [139]. Heterozygous mice survive the perinatal period but develop progressive, age-related increases in plasma ALT levels, associated with hepatic steatosis, oxidative stress, and mitochondrial degeneration. MPT heterozygous mice do not develop liver necroinflammation or hepatic fibrosis, however [140].

Other Rodent Genetic Models

PTEN Null Mice

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) encodes a lipid phosphatase, with its main substrate being phosphatidylinositol-3,4,5-triphosphate (PI3P). It acts as a tumor suppressor by inhibiting the PIP3 kinase and serine-threonine protein kinase B (PkB or Akt) pathways [6]. Mice that are global knockouts (KO) for PTEN are not viable, dying early during embryonic development [141]. Liver-specific KO, $PTEN^{loxP/loxP}$, and Alb-Cre^{+} are viable but develop NAFLD with increased synthesis of fatty acids and glycogen. Contrary to human NAFLD, these mice exhibit liver hypersensitivity to insulin [141]. Liver-specific PTEN KO mice develop progressive liver disease, with macrovesicular steatosis by week 10 of age; NASH with hepatocellular ballooning, Mallory-Denk bodies, and sinusoidal fibrosis by week 40; and hepatocellular carcinoma with a penetrance of 47 % at week 44 and 100 % at weeks 74–78 [35, 142].

Methionine Adenosyltransferase 1A-Deficient Mice

Methionine adenosyltransferase (MAT) is the enzyme that catalyzes the conversion of methionine and ATP in SAMe. The isoform 1A is liver specific [11]. KO mice have impaired antioxidant defense and perturbations of lipid metabolism

[143]. Those mice develop spontaneous NASH with fibrosis at 8 months of age. Although they are hyperglycemic, their insulin levels are normal and they do not develop other features of the metabolic syndrome. MAT1A KO mice exhibit a higher susceptibility to development of liver tumors [144].

Cystathionine β-Synthase-Deficient Mice

Cystathionine β-synthase (CBS) deficiency causes severe hyperhomocysteinemia. Homocysteine may be recycled in methionine. Alternatively, it can be converted in cystathionine, which can then be metabolized to cysteine, an important substrate for the synthesis of the antioxidant glutathione [145]. As such, hyperhomocysteinemia induces liver injury by oxidative stress. In addition, the condition induces ER stress and results in enhanced lipid biosynthesis and, hence, liver steatosis [146]. Homozygous KO mice for CBS develop hepatic steatosis and fibrosis at 15 days old on standard rodent diet and usually die before 1 month of age [145]. However, if fed a diet supplemented with choline (1.592 g/kg), the mice survive through adulthood. They develop progressive liver disease, with steatosis, mild inflammation, and fibrosis around vessels by the age of 8 weeks, pericellular fibrosis at 12 weeks, and mild portal fibrosis with worsening fibrosis at 32 weeks [145].

Interleukin (IL)-6 Deficiency

Human NAFLD and HFD-induced NAFLD in rodents associate with increased levels of IL-6, an important inflammatory cytokine [147]. IL-6 also has beneficial actions, however, including induction of compensatory protective mechanisms that guard against energy surplus. IL-6 KO mice develop spontaneous obesity, NASH, and insulin resistance at 4 months of age [148]. When IL-6 KO mice are fed HFD for 12 weeks, they develop worse liver injury, necroinflammation, and insulin resistance than HFD-fed wild-type mice, despite similar weight gain and ectopic fat accumulation [148, 149]. Mice deficient in IL-10, an anti-inflammatory cytokine, develop a huge liver inflammatory response with increase in IL-6 and downstream activation of STAT-3 when fed HFD for 12 weeks. Despite this increased inflammatory response, these mice develop less steatosis and less hepatocellular damage than wild-type controls, suggesting a "disconnect" between IL-6-mediated inflammation and fatty liver damage. This concept is supported by evidence that deletion of IL-6 or STAT-3 restored HFD-induced steatosis and hepatocellular damage in IL-10-deficient mice. Surprisingly, the liver inflammatory response also worsened in the absence of IL-6/STAT-3. In the liver, IL-6-mediated induction of STAT-3 was shown to downregulate lipogenic genes and upregulate genes associated with fatty acid oxidation [149]. A recent study showed that increased production of IL-6 might be a compensatory response to obesity that skews macrophages toward a less-inflammatory (i.e., M2) phenotype, thereby limiting the development of insulin resistance. The authors took advantage of specific KO mice to IL-6 receptor α chain in myeloid cells and submitted them to HFD. Compared to wild-type controls, those mice showed higher insulin resistance in adipose tissue and liver; higher systemic, hepatic, and adipose tissue inflammatory response; and a shift toward M1 phenotype [150].

NEMO-Deficient Mice

The IkB kinase (IKK) is essential for activation of the survival factor nuclear factor kappa B (NFkB), which regulates cellular responses to inflammation. Mice with global KO of the IKK subunit NEMO develop massive hepatocyte apoptosis and die during embryonic development. However, liver-specific conditional KO mice develop spontaneous NASH, with steatosis, inflammation, hepatocellular ballooning, apoptosis, and fibrosis, as young as 8 weeks of age. At 12 months they develop hepatocellular carcinoma with 100 % penetrance [151]. Interestingly, hepatocyte apoptosis seems related to activation of DR5 signaling through bile acid deregulation in Alb-Cre, IKK-deleted mice because treating these mice with nor-ursodeoxycholic acid decreased liver apoptosis, inflammation, and fibrosis [152].

Summary

There are a large number of genetic models of NAFLD/NASH. In general, the ones that associate with obesity and the metabolic syndrome do not develop severe NASH or fibrosis. In order to achieve those phenotypes, a second stimulus is necessary, such as exposure to either MCD diet or HFD. Leptin-deficient ob/ob mice, one of the most used models, remain resistant to fibrosis even when subjected to added dietary challenges, demonstrating the importance of leptin signaling in liver fibrogenesis.

There are some models of spontaneous NASH that progress to significant fibrosis and hepatocellular carcinoma over time. Unfortunately, those models generally do not exhibit the metabolic background characteristic of human NAFLD.

Another disadvantage of genetic animal models is that proving causality mandates difficult breeding strategies, considerable time, and expense (Table 6.2).

Non-rodent Models of NAFLD/NASH

Opossum Model

Gray short-tailed opossum (*Monodelphis domestica*), when fed a high-fat (19 % calories from fat), high cholesterol (0.7 %) diet for 24 weeks, may develop severe hypercholesterolemia (40-fold increase in VLDL and LDL) or be resistant to it, being classified as high or low responders, respectively. This diet response associates with a variant in the ABCB4 gene [153]. High responders also develop typical features of fibrosing NASH, with elevation of plasma aminotransferases, steatosis, lobular inflammation, hepatocellular ballooning, and perisinusoidal/periportal fibrosis [154].

Ossabaw Pigs

Mini-pigs residing in the Ossabaw Island exhibit a thrifty genotype, developing obesity, insulin resistance, hypertension, and dyslipidemia when fed a high-caloric/atherogenic diet. Thus, these animals are a good model to study the metabolic syndrome and type 2 diabetes mellitus [155]. When the pigs are fed a modified atherogenic diet, with lower choline content (700 ppm versus 1500 ppm in regular diet), they develop liver injury similar to human NASH, with macro- and microvesicular steatosis, inflammation with foamy Kupffer cells, extensive hepatocellular ballooning, and pericellular/perisinusoidal fibrosis. They also exhibit the usual adipokine pattern observed in human NAFLD, with low levels of serum adiponectin and high levels of leptin and TNF-α [156].

Fish Models

Fish models of NAFLD have several advantages over more widely used small mammal models. Namely, fish are less expensive, have shorter generation times, and are highly fertile. Thus, fish are efficient tools for screening studies. Fish larvae also are optically clear, allowing visualization of internal organs without surgery. Also, fat and specific proteins can be visualized in live fish using fluorescent tags [157]. However, fish liver structure is different from mammals, and its small size makes molecular analysis of tissue more difficult.

Two fish models have been used to study metabolic diseases and NAFLD, zebra fish (*Danio rerio*) and the ricefish medaka (*Oryzias latipes*). When fed HFD for 12 weeks, medaka develop hyperlipidemia, hyperglycemia, and liver disease characterized by steatosis, hepatocellular ballooning, apoptosis, and fibrosis [158–160]. A diet-induced obesity (DIO) model in zebra fish overfed with *Artemia* (a living prey with high amount of fat) has also been used to study hepatic steatosis. This model exhibits overexpression of inflammatory cytokines (IL-6 and IL-1β) and lipolytic transcription factors (SREBP-1 and PPAR-γ) in the liver [161]. Treating zebra fish larvae from day 3 to 5 postfertilization elicits an acute phase response, ER stress, hepatomegaly with steatosis, and hepatic stellate cell proliferation and activation [161].

Mutant fish with disrupted ER function, or deregulated metabolism of methionine, phospholipids, and lipids also manifest hepatic steatosis and variable degrees of ER stress but lack evidence of inflammation/NASH [162–166]. Several other transgenic fish models demonstrated increased hepatic lipogenesis with hepatic steatosis [167–169], and spontaneous degeneration to hepatocellular carcinoma has been reported to occur in one of these [167].

Conclusion

The quest for the ideal animal model for human NAFLD/NASH continues. Nevertheless, a variety of animal models are currently available. Some of these are more ideal for modeling the physiology of liver steatosis in the context of the metabolic syndrome, while others better model hepatic inflammation and fibrosis. Ultimately, model selection should be guided by the purpose of the study.

Rodents are by far the most used animals to model NAFLD, since they provide a good balance between cost, time necessary for breeding and intervention, and amount of tissue samples that can be obtained. Mice are particularly amenable for genetic manipulation, allowing for causality studies. Two strategies have been used to evoke NAFLD/NASH, dietary and genetic. Regarding the former, a diet deficient in methionine and choline is the one that produces more severe liver injury, namely, inflammation and fibrosis, in a very reproducible way and after the shortest duration of diet exposure (i.e., 1–2 months). However, it does not induce the metabolic syndrome, and animals lose considerable weight. High-fat diets better model the metabolic syndrome and can also induce fibrosing NASH if they contain high contents of saturated fat and cholesterol and are supplemented with high-fructose corn syrup equivalents. However, a much longer duration of feeding is required, ideally starting as soon as possible after weaning and continuing for an additional 4–6 months. Another disadvantage of this time-consuming and costly approach is that it generally induces relatively mild liver injury and fibrosis, and there appears to be considerable inter-animal variability with regard to the severity of liver injury, even within the same feeding cohort. Several genetic mouse models exhibit obesity, diabetes mellitus, and NAFLD, the most used ones being ob/ob and db/db mice. However, to induce severe NASH and particularly hepatic fibrosis in these strains, a second dietary "hit" is necessary, such as feeding a methionine- and choline-deficient diet. Even with this added dietary challenge, however, only mild liver fibrosis develops in ob/ob mice. Other genetically abnormal mice manifest spontaneous NASH with fibrosis and even hepatocellular carcinoma. However, similar to MCD diet-fed wild-type mice, these genetic models of "spontaneously" progressive NASH are not naturally obese or insulin resistant. More recently, non-rodent models have been used to study NASH, such as opossum and mini-pigs. However, data on these models is still scarce, and they are more expensive than rodent NAFLD/NASH models. Increasingly, fish are being used to model NAFLD/NASH. Fish provide a very useful model to screen for potential genetic defects associated with disease and for therapeutic approaches, but results still need to be confirmed in mammals.

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Genetic Basis of Alcoholic and Nonalcoholic Fatty Liver Disease

7

Silvia Sookoian and Carlos Jose Pirola

Abbreviations

AFLD	Alcoholic fatty liver disease
ALT	Alanine aminotransferase
GGT	Gamma-glutamyl transpeptidase
GWAS	Genome-wide association studies
MetSyn	Metabolic syndrome
miRNA	*microRNA*
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
PNPLA3	Patatin-like phospholipase domain-containing protein 3
SNP	Single-nucleotide polymorphism
TAG	Triacylglycerol

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Abnormal Deposition of Triglycerides in the Liver in Nonalcoholic Fatty Liver Disease and Alcoholic Fatty Liver Disease: A Common Underlying Pathogenic and Genetic Background

The abnormal deposition of triglycerides in the liver is known as fatty liver. While the transformation of fatty liver may occur owing to a number of factors [1], it is often associated either with metabolic risk factors of the metabolic syndrome (MetSyn) in the absence of alcohol consumption (nonalcoholic fatty liver disease, NAFLD) [2], including insulin resistance and obesity, or with chronic alcohol consumption (alcoholic fatty liver disease, AFLD) [3].

NAFLD and AFLD progress from a mild clinical form characterized by liver fat accumulation (simple steatosis) to a more severe diagnosis associated with either liver inflammation and/or fibrosis (steatohepatitis) [2, 3]. Despite the causative insult of NAFLD and AFLD being different, both diseases share similar underlying pathogenic mechanisms [4] and a common underlying genetic component [5, 6], summarized in Fig. 7.1.

The knowledge regarding the pathogenesis NAFLD and its genetic component has significantly increased in the last 10 years when clinicians, gastroenterologists, and hepatologists started to realize the harmful health consequences of the obesity and type 2 diabetes pandemic that

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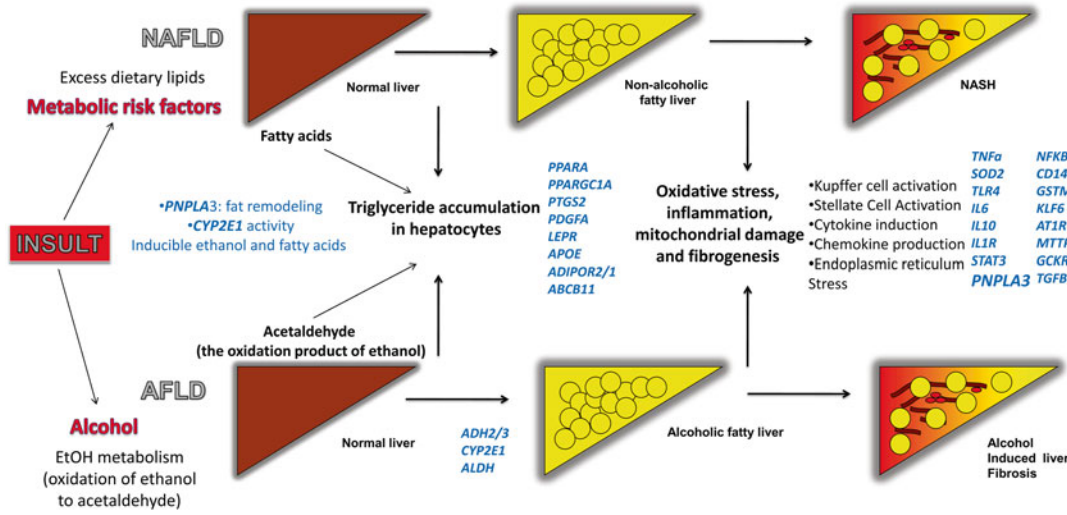

Fig. 7.1 NAFLD and AFLD share similar underlying pathogenic mechanisms and a common underlying genetic component. The *cartoon* depicts the disease progression of NAFLD and AFLD from normal liver to steatohepatitis and advanced disease with or without liver fibrosis. Liver triglyceride accumulation is a common underlying process in both diseases, and the transition from simple steatosis to progressive disease involves both NAFLD and AFLD oxidative stress, inflammation, mitochondrial damage, and fibrogenesis. The genes reported to be involved in these disease pathogenic pathways are highlighted in *light blue*. *PNPLA3*, patatin-like phospholipase domain-containing protein 3; *CYP2E1*, cytochrome P450, family 1, subfamily A, polypeptide 2; *PPARA*, peroxisome proliferator-activated receptor alpha; *PPARGC1A*, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; *PTGS2*, prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase); *PDGFA*, platelet-derived growth factor alpha polypeptide; *LEPR*, leptin receptor; *APOE*, apolipoprotein E; *ADIPOR2/1*, adiponectin receptor 2/1; *ABCB11*, ATP-binding cassette, subfamily B (MDR/TAP), member 11; *TNFa*, tumor necrosis factor a; *SOD2*, superoxide dismutase 2, mitochondrial; *TLR4*, toll-like receptor 4; *IL-6*, interleukin 6 (interferon, beta 2); *IL-10*, interleukin 10; *IL-1R*, interleukin 1 receptor; *STAT3*, signal transducer and activator of transcription 3 (acute-phase response factor); *NFKB*, nuclear factor of kappa light polypeptide gene enhancer in B cells; *CD14*, monocyte differentiation antigen CD14; *GSTM1*, glutathione S-transferase mu 1; *KLF6*, Kruppel-like factor 6; *AT1R*, angiotensin II receptor, type 1; *MTTP*, microsomal triglyceride transfer protein; *GCKR*, glucokinase (hexokinase 4) regulator; *TGFB1*, transforming growth factor, beta 1; *ADH2/3*, alcohol dehydrogenase 2/3; *ALDH*, aldehyde dehydrogenase 2 family (mitochondrial)

affected Western countries initially but now has expanded globally [7].

During the 1980s, Ludwig regarded nonalcoholic steatohepatitis (NASH) as *a poorly understood and hitherto unnamed liver disease that histologically mimics alcoholic hepatitis and that also may progress to cirrhosis* [8]. Nevertheless, "fatty liver" is not an emerging disease for hepatologists; on the contrary, fatty liver has been a "hub" of intense and fructiferous research for over 30 years. For example, in 1961, Charles Lieber observed that fatty infiltration of the liver was a common finding in alcoholic patients [9]. Interestingly, Lieber showed that unlike adipose tissue, ethanol is oxidized by alcohol dehydrogenase (ADH) in the liver, and he suggested that the excess ethanol in the liver leads to fatty acid biosynthesis [9] (Fig. 7.2). Four years later, Lieber performed studies in patients who had a history of alcohol consumption by doing liver biopsies during and after alcohol ingestion [10]; remarkably, Lieber's observations showed that the steatosis seemed to depend on both the dose and the duration of ethanol intake. It was not possible to establish a threshold for the production of fatty liver by ethanol because some individuals had liver damage after exposure to high amounts of alcohol while others did not [10].

This observation can be translated into a modern concept of the etiology of human diseases. This concept assumes that a genetic component that illustrates the interindividual differences in

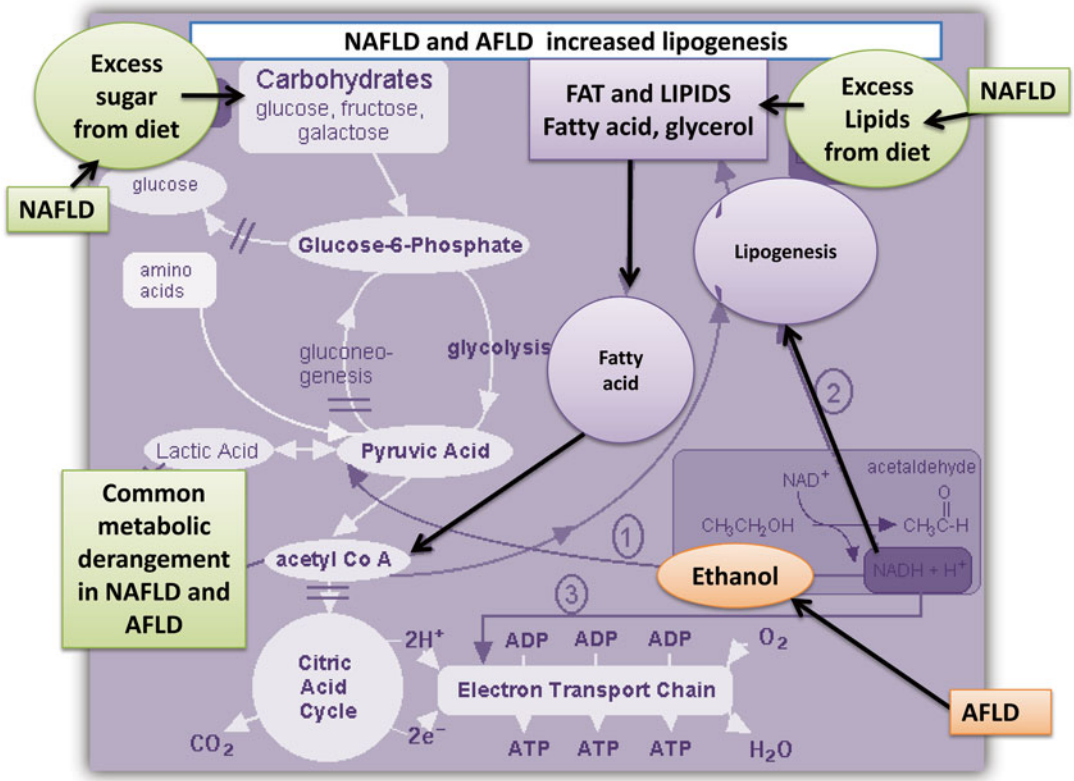

Fig. 7.2 NAFLD and AFLD are associated with increased lipogenesis. The *cartoon* illustrates the metabolic pathway associated with increased lipogenesis in NAFLD (increased energy from diet) and AFLD (metabolism of ethanol that feeds the fatty acid metabolism cycle by converting to acetaldehyde)

response to similar insults can explain the disease susceptibility of complex diseases.

On the other hand, NAFLD and AFLD share not only the degree of histological changes, including the presence of lobular inflammation, morphological changes in liver mitochondria, perivenular and perisinusoidal fibrosis, and even hepatocellular ballooning [11, 12], but also, as we have shown recently, they seem to share similar underlying molecular disease mechanisms. From a genetic point of view, they are difficult to distinguish from each other [4]. More importantly, hepatic fat accumulation in NAFLD and AFLD is connected by a common metabolic pathway (Fig. 7.2) that involves enzymatic reactions that impact on the citric cycle [13–15]. Furthermore, as ethanol increases the rate of synthesis of fat [16], an increasing number of lipogenic enzymes show increased transcriptional activity in both NAFDL and AFLD [16].

In this chapter, we review the current knowledge and recent insights regarding the genetic basis of NAFLD and AFLD in an integrative approach to understand the role of the genetic component in the susceptibility of abnormal liver fat accumulation and the progression of liver disease.

The Genetic Component of NAFLD and AFLD and the Role of *PNPLA3* Gene on Liver Fat Accumulation

The genetic component of polygenic human diseases can be studied by two main approaches: candidate gene association studies, which are, in general, hypothesis driven, and genome-wide

association studies (GWAS). Although advances in genotyping technology and information generated by GWAS have expanded our knowledge about gene variants associated with complex diseases, including NAFLD and AFLD, many *important questions* remain unanswered. For example, single-nucleotide polymorphisms (SNPs) identified by GWAS are not necessarily involved in the disease biology because they do may represent the causal variant.

A growing body of evidence indicates that NAFLD [5] and AFLD [6] develop from a complex process in which many factors, including genetic susceptibility and environmental insults, are involved. Data from the first GWAS on NAFLD [17] have significantly contributed to our knowledge of the genetic component of fatty liver, as it stemmed the search for replication of its findings in patients with a myriad of liver diseases, including AFLD [18].

Nowadays, the nonsynonymous SNP, rs738409 C/G, of *PNPLA3* (patatin-like phospholipase domain-containing protein 3, also known as adiponutrin or calcium-independent phospholipase A2-epsilon), encoding an amino acid substitution I148M, is regarded as the major genetic component of hepatic triglyceride accumulation and fatty liver disease, including NAFLD [19] and AFLD [20]. In fact, the risk effect of the rs738409 on developing fatty liver in the context of NAFLD is perhaps one of the strongest ever reported for a common variant modifying the genetic susceptibility for complex diseases (5.3 % of the total variance) [19].

In addition, the rs738409 is not only significantly associated with the accumulation of fat in the liver but also with the histologic disease severity and progression of NAFLD (OR 1.88 per G allele; 95 % CI = 1.03–3.43; $p < 0.04$) [19] and the development of cirrhosis in AFLD (OR = 2.08; 95 % CI = 1.15–3.77; $p = 0.02$) [21].

Indeed, the rs738409 variant not only modifies the biology of NAFLD but also has a considerable impact on the genetic susceptibility to alcoholic liver disease (ALD) [20–22] and hepatitis C- [23] and hepatitis B-induced fatty liver, and it has also been associated with hepatocellular carcinoma occurrence among patients with cirrhosis [24–26], indicating that these diverse liver diseases may share common pathophysiological pathways associated with accumulation of the fat in the liver.

PNPLA3 is a multifunctional enzyme with both triacylglycerol (TAG) lipase and acylglycerol O-acyltransferase activity that participate in TAG hydrolysis and the acyl-CoA-independent transacylation of acylglycerols [27]. Interestingly, nutritional factors, including oleic acid, C18:2 fatty acid, palmitic acid, glucose, insulin, and lactone, induce *PNPLA3* [27]. In addition, the promoter activity of *PNPLA3* is upregulated by glucose concentrations in a dose-dependent manner [28].

The basic function of *PNPLA3* was explored in adipose tissue initially and can be summarized as follows: (1) it is strongly associated with membranes and with lipid droplets; (2) it has triacylglycerol lipase activity that mediates triacylglycerol hydrolysis in adipocytes; (3) it may be involved in the balance of energy usage/storage in adipocytes; (4) it participates in triacylglycerol hydrolysis and the acyl-CoA-independent transacylation of acylglycerols, thereby facilitating energy mobilization and storage in adipocytes [29]; (5) it has lipid hydrolase with an unusual folding topology that differs from classical lipases; (6) its function may be related to some regulatory aspect of the pathway of lipogenesis or lipolysis; and (7) it plays a role in the hydrolysis of glycerolipids [30]. Figure 7.3 depicts the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway of glycerolipid metabolism, in which *PNPLA3* involvement is highlighted. Nevertheless, the role of *PNPLA3* in the liver seems to be broader than adipose tissue, involving hepatocyte triacylglycerol remodeling [18, 30] and global metabolic changes [31].

Whether the rs738409 variant is associated with a gain or loss of function has been a matter of debate [18]. For example, Kumari et al. reported that *PNPLA*3 promotes cellular lipid synthesis by converting lysophosphatidic acid into phosphatidic acid; hence, the I148M substitution promotes hepatic lipid synthesis due to a gain of function [32]. Conversely, in vitro examination of *PNPLA3* enzymatic activity showed that the wild-type protein shows a predominant lipase activity with

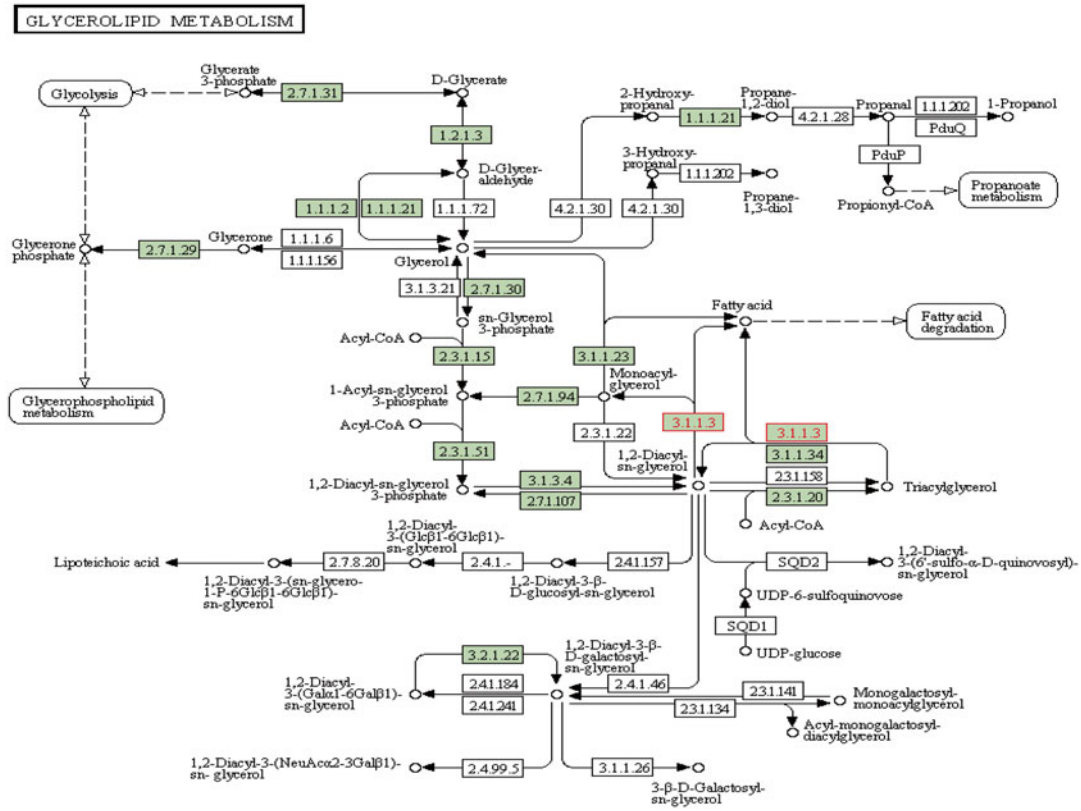

Fig. 7.3 The KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway of glycerolipid metabolism. The *pink* highlights the 3.1.1.3 enzymatic reactions involved in acylglycerol degradation, in which *PNPLA3* is involved

mild lysophosphatidic acid acyl transferase activity while the I148M mutation results in a loss of function of both these activities [33]. Likewise, more recently, Smagris and coworkers explored the effect of introducing a methionine codon at position 148 of the mouse *PNPLA3* gene; when mice were challenged with a high-sucrose diet, their liver fat levels increased two to threefold compared with wild-type littermates [34]. The authors also showed that the catalytically inactive *PNPLA3* on the surfaces of lipid droplets was associated with liver fat accumulation [34].

Finally, a recent study uncovered the functional role of *PNPLA3* on liver metabolism by performing high-throughput metabolic profiling of *PNPLA3* siRNA silencing and overexpression of wild-type and mutant Ile148Met variants (isoleucine/methionine substitution at codon 148) in Huh-7 cells [31]. Of note, the silencing of *PNPLA3* was associated with a global perturbation of Huh-7 hepatoma cells that resembled a catabolic response associated with protein breakdown; a significant decrease in amino- and gamma-glutamyl amino acids and dipeptides and a significant increase in cysteine sulfinic acid, myo-inositol, lysolipids, sphingolipids, and polyunsaturated fatty acids were observed [31]. On the other hand, overexpression of the *PNPLA3* Met148 variant mirrored many of the metabolic changes observed during gene silencing but in the opposing direction. Interestingly, overexpression of the *PNPLA3* Met148 variant was associated with a 1.75-fold increase in lactic acid in comparison with the empty vector, suggesting a shift to anaerobic metabolism and mitochondrial dysfunction. Together, these results suggest a critical role of *PNPLA3* in the modulation of liver metabolism beyond its classical participation in triacylglycerol remodeling [31] and could explain the role of *PNPLA3* in disease progression.

GWAS on NAFLD and the Genetic Risk of Disease Progression

The first GWAS on NAFLD was a genome-wide survey of nonsynonymous sequence variations encompassing 9229 SNPs in a multiethnic population-based study [17]. The use of the GWAS strategy in the search for the genetic component of NAFLD was followed by other reports that included different populations, study designs, sample sizes, and approaches for the characterization of the main liver phenotype, for example, female adults with NAFLD diagnosed by liver biopsy [35], exploration of the heritability of hepatic steatosis at the population level with computed tomography [36], a combined approach of CT and alanine aminotransferase (ALT) levels as a surrogate of disease severity [37], exploration of the genetic risk in Asian-descent patients [38, 39], and liver fat content in extremely obese individuals [40].

It is also important to highlight that the coverage of SNPs by the abovementioned GWAS was not uniform in terms of the explored variants. In addition, it varied from a GWAS analysis of 12,138 nonsynonymous sequence variations from dbSNP and the Perlegen SNP database [17] to commercial platforms, such as HumanCNV370-Quadv3 BeadChip (coverage: 373,397 SNPs) [35] or Illumina Human 610-Quad BeadChip (coverage: 484,751 SNPs), meta-analysis, and GWAS association data of large consortiums that used the Affymetrix 6.0 or Illumina platform [36] and imputed SNPs [37].

Finally, the GWAS strategy was also used to explore the genetic locus that influenced liver enzyme levels in the population, including ALT [41, 42]. Figure 7.4 depicts a summary of the latest GWAS on NAFLD and ALT levels. Of note, these GWAS uncovered loci whose function is diverse but interesting in the context of NAFLD. For instance, *PPP1R3B* (protein phosphatase 1,

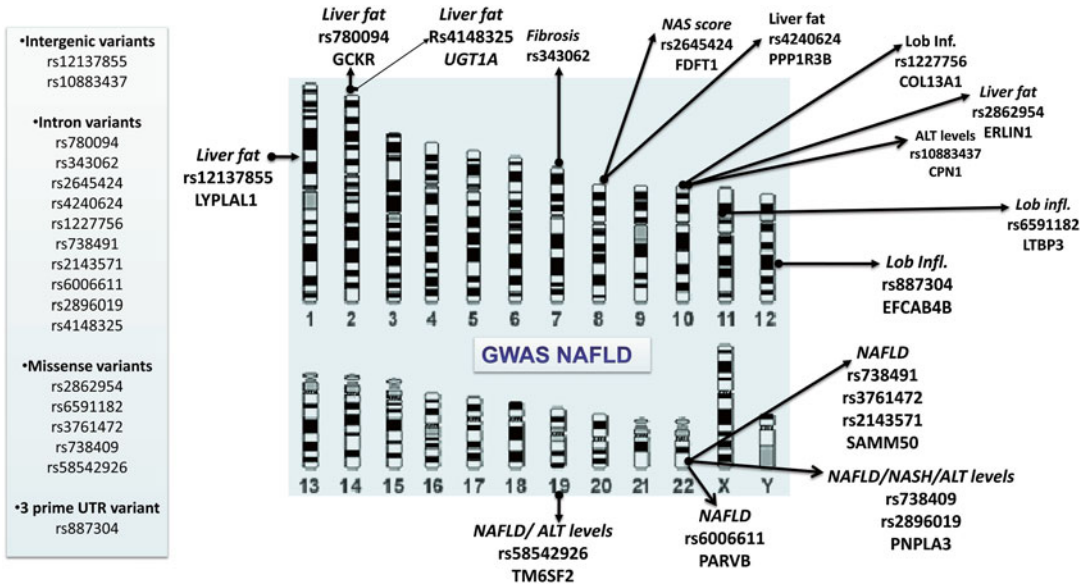

Fig. 7.4 GWAS on NAFLD: Summary representation of variants significantly associated with NAFLD, NASH, and plasma levels of alanine aminotransferase (ALT). The illustration depicts the chromosome localization of significantly associated SNPs according to the main NAFLD phenotypes. *LYPLAL1*, lysophospholipase-like 1; *GCKR*, glucokinase (hexokinase 4) regulator; *COL13A1*, collagen, type XIII, alpha 1; *PPP1R3B*, protein phosphatase 1, regulatory subunit 3B; *ERLIN1*, ER lipid raft-associated 1; *EFCAB4B*, EF-hand calcium-binding domain 4B; *TM6SF2*, transmembrane 6 superfamily member 2; *PARVB*, parvin beta; *PNPLA3*, patatin-like phospholipase domain-containing protein 3; *SAMM50*, sorting and assembly machinery component 50 homologue (*S. cerevisiae*); *LTBP3*, latent transforming growth factor beta-binding protein 3; *FDFT1*, farnesyl-diphosphate farnesyltransferase 1; *CPN1*, carboxypeptidase N, polypeptide 1; *UGT1A*, glucuronosyltransferase 1 family

regulatory subunit 3B) is associated with the regulation of glycogen synthesis in liver or skeletal muscle; *FDFT1* (farnesyl-diphosphate farnesyltransferase) is involved in cholesterol biosynthesis; *ERLIN1* (ER lipid raft-associated 1) mediates the endoplasmic reticulum-associated degradation; *LTBP3* (latent transforming growth factor beta) plays a structural role in the extracellular matrix; and *PARVB* (parvin beta) plays a role in cytoskeleton organization and cell adhesion. Conversely, certain loci, such as *CPN1* (carboxypeptidase N, polypeptide 1), *NCAN* (neurocan), and *EFCAB4B* (EF-hand calcium-binding domain 4B), whose biological function seems to be distant from the pathogenesis of NAFLD are still worth being explored from the mechanistic point of view to reveal their role in disease susceptibility. In fact, rs2228603 in *NCAN* locus is an interesting example of misinterpretation of casual variants in NAFLD-GWAS [36]. While no functional study was done on the abovementioned *NCAN* variant, it was speculated that rs2228603[T] allele is a risk factor for liver inflammation and fibrosis, suggesting that this locus is responsible for progression from steatosis to steatohepatitis [43]. Furthermore, despite the biological plausibility of a putative role of *NCAN* in NAFLD being hard to support as this gene codes for a chondroitin sulfate proteoglycan expressed primarily in nervous system, it was speculated that rs2228603 is associated with a "brain-liver axis" that, when deregulated, increases the risk for NAFLD [43]. Remarkably, recently findings from an exome-wide association study [44], followed by replication studies in different population around the world [45–49], have definitively showed that the causal variant in the multigene locus named *NCAN/TM6SF2/CILP2/PBX4* is the nonsynonymous variant located in the *TM6SF2* (transmembrane 6 superfamily member 2) gene, the rs58542926 encoding an amino acid substitution p.Glu167Lys (E167K). The initial study of Kozlitina et al. showed that rs58542926 was significantly associated with hepatic triglyceride content (HTGC) as measured by proton magnetic resonance spectroscopy (H-MRS). The authors showed that the effect of the rs58542926 on HTGC was independent of the effect mediated by the rs738409, obesity, insulin resistance as assessed by HOMA-IR, or alcohol intake [44].

Subsequent studies showed conflicting results as some [45, 47–49] but not all studies [46, 50] showed a significant association with fatty liver or histological steatosis, and the association with liver fibrosis reported by one large study [46] remains to be confirmed as most of the studies showed that the association does not resist adjustment by NASH [45] or was not significant [47, 50]. In fact, a recent study on chronic hepatitis C showed that while *TM6SF2*-E167K variant is an independent predictor of liver, no difference in necroinflammatory or fibrosis scores was found among carriers and noncarriers of the risk allele [51]. Finally, functional studies on *TM6SF2* gene demonstrated that this locus and the mentioned variant are relevant on NAFLD disease biology. For instance, Kozlitina et al. showed in vitro that murine hepatoma cells expressing the Lys167-TM6SF2 (E variant) protein have reduced expression levels compared with the wild type [44], and Mahdessian demonstrated that TM6SF2 is localized in the endoplasmic reticulum and the ER-Golgi intermediate compartment of human liver cells [52]. Moreover, to understand whether the rs58542926 genotypes have any effect on human NAFLD, our group explored the level of liver *TM6SF2* expression in subjects with NAFLD at different stages of disease severity [47]. Interestingly, we observed that TM6SF2 protein expression was significantly reduced in the liver of patients with NAFLD; reduced expression of liver TM6SF2 was associated with a high degree of steatosis and NAS score. In addition, we noted that liver TM6SF2 immunoreactivity was reduced in carriers of the NAFLD-risk T allele (Lys167); allelic-specific expression analysis of cDNA isolated from the liver tissue confirmed that expression levels of rs58542926-T are about 56 % of that of the C allele. Taken together, these findings suggest that the *TM6SF2-NAFLD-risk* T allele is associated with decreased gene and protein expression in the liver of affected patients [47].

Of note, as we initially noticed, the *TM6SF2-rs58542926* presents a clinical paradox as the C (Glu167) allele is associated consistently with

increased cardiovascular risk by increasing circulating LDL cholesterol [53] and replicated by others [44, 45, 47] but the other T allele (Lys167) is associated putatively with NAFLD and NASH. Nevertheless, it is important to note that rs58542926 is a low-frequency variant with a rather modest putative protective and risk effect on CVD and NAFLD, respectively. This observation supports the concept that other than "common-frequent" variants may contribute to the heritability of NAFLD and may also explain, at least in part, the missing heritability problem.

Finally, the association of the rs58542926 and the level of serum transaminases remain to be elucidated because the association was not replicated consistently in different cohorts as we recently summarized [47].

Evidence About the Heritability of NAFLD and Related Disease Phenotypes

The search of the genetic component of complex diseases is enriched by research studies that explore the hereditability of a given disease by performing familial aggregation studies. This kind of study is focused on determining whether the index cases (probands) have relatives that cluster similar phenotypes; hence, familiar aggregation studies are devoted primarily to seeking whether having relatives with disease increases one's risk of that disease.

Struben and colleagues provided the first evidence on the heritability of NAFLD when they examined the familial pattern of cryptogenic cirrhosis by reviewing the family history of patients with NASH with and without cirrhosis or cryptogenic cirrhosis to assess how frequently their relatives were afflicted with these disorders [54]. The authors included 18 members of eight kindreds containing two or more afflicted members and observed that NASH coexisted within four kindreds; the pattern of afflicted patients included mother–daughter, sister–sister, sister–brother, father–daughter, and male–female cousins [54].

Schwimmer et al. performed a familial aggregation study of fatty liver in overweight children with and without NAFLD and demonstrated that fatty liver was present in 17 % of siblings and 37 % of parents of overweight children without NAFLD [55]. Abdelmalek et al. described familial aggregation of insulin resistance in first-degree relatives of patients with NAFLD [56], and Loomba reported similar findings in patients from the Nonalcoholic Steatohepatitis Clinical Research Network that explored the family history of type 2 diabetes in subjects with NASH and NAFLD and concluded that family history of diabetes, especially among nondiabetics, is associated with NASH and fibrosis in NAFLD [57].

Finally, an elegant study on the University of California at San Diego twin cohort with 362 twins showed that plasma gamma-glutamyl transpeptidase (GGT) is a heritable trait and that GGT shares significant genetic covariance with uric acid, insulin, HOMA-IR, triglycerides, and blood pressure [58].

Likewise, unpublished data from the "The Genetics of NAFLD in Twins Consortium" led by Rohit Loomba and colleagues demonstrated that while both hepatic steatosis and hepatic fibrosis are heritable traits, they appear to have distinct basis for their genetic susceptibility. Notably, genetic covariance assessment revealed a significant association between hepatic steatosis and BMI and hyperinsulinemia and between hepatic fibrosis and HbA1c (Rohit Loomba, unpublished personal communication).

Ethanol Metabolism and Genetic Variants Influencing Alcoholic Liver Disease

Once ingested, several enzymatic and nonenzymatic mechanisms in the liver and other organs like the stomach metabolize ethanol. The first step of ethanol metabolism is its oxidation into acetaldehyde, which is oxidized subsequently to acetate through several enzymatic reactions, including aldehyde dehydrogenase. The alcohol dehydrogenase family is composed of by at least seven genes and comprises at least five classes [59]; isozymes are distributed differentially in tissues, with most classes exhibiting the highest

activity in liver. Alcohol dehydrogenase (ADH1), which consists of several homo- and heterodimers of alpha, beta, and gamma subunits, catalyzes the oxidation of alcohols to aldehydes; three genes encoding alpha, beta, and gamma subunits are organized tandemly in a genomic segment as a gene cluster. ADH1A is active in the liver in early fetal life but only weakly active in adult liver. Alcohol dehydrogenase 1B (ADH1B, class I) beta polypeptide is a protein that exhibits high activity for ethanol oxidation and plays a major role in ethanol catabolism. ADH1C is a paralog of ADH1A and B. ALDH2 (aldehyde dehydrogenase 2 family) is the second enzyme of the major oxidative pathway of alcohol metabolism, with two major liver isoforms of aldehyde dehydrogenase, cytosolic and mitochondrial. ADH4 (alcohol dehydrogenase 4 (class II), pi polypeptide) exhibits a high activity for oxidation of long-chain aliphatic alcohols and aromatic alcohols and is less sensitive to pyrazole.

Genetic polymorphisms in the ADH and ALDH families of genes are responsible for the high individual variability in ethanol metabolism, as gene variants determine the level of acetaldehyde accumulation after alcohol consumption [6, 60, 61]. Around 50 % of East Asian subjects carry an inactive ALDH2 gene and exhibit acetaldehyde accumulation after alcohol consumption. Interestingly, compared with wild-type mice, ethanol-fed ALDH2–/– mice had higher levels of malondialdehyde-acetaldehyde (MAA) adduct and greater hepatic inflammation, with higher hepatic interleukin (IL)-6 expression but lower levels of steatosis and serum alanine aminotransferase (ALT) [62].

Another important family of genes that mediates ethanol metabolism is the cytochrome P450 superfamily of enzymes that are monooxygenases, which catalyze many reactions involved in drug metabolism and the synthesis of cholesterol, steroids, and lipids. Cytochrome P450, family 2, subfamily E, polypeptide 1 (CYP2E1) localizes to the endoplasmic reticulum and is induced by ethanol; thus the enzyme metabolizes both endogenous substrates, such as ethanol, acetone, and acetal as well as exogenous substrates. A recent meta-analysis showed that CYP2E1 might be significantly associated with the development of steatosis, hepatitis, and fibrosis in patients with AFLD [63]. It is worth mentioning that there are no published GWAS in AFLD, though two efforts are ongoing—one is a multinational GWAS of alcoholic cirrhosis and another is a US-based GWAS of acute alcoholic hepatitis.

The Role of Epigenetic Mechanisms in the Development and Disease Progression of NAFLD and AFLD

Genetic factors other than DNA variation are likely to play an important role in the etiology of complex diseases, specifically epigenetic modifications. By definition, epigenetic factors, which are the most important as described below, are DNA methylation and covalent histone modifications typically at lysine (K) in their N terminal regions. These are modifiers of gene expression that without altering the DNA sequence itself, are capable of self replication through cell mitosis and even of being transmitted to the next organismal generation. Actually, the most interesting points about epigenetic modifications are that they are crucial during development and they are potentially modified and disrupted by environmental influences, mainly dietary and behavioral habits and therapeutic intervention.

Cytosine (C) methylation (5-methylcytosine) is a common epigenetic modification, where a C is adjacent to a guanine (G) nucleotide (CpG). In the whole mammalian genome, around 5 % of cytosines are methylated, typically outside of the so-called CpG islands (regions of typically 300–3000 bp in length with a high content of CpG and C/G %). Nearly 50–60 % of genes have CpG-rich islands in their 5′ untranslated regions near or in the promoters. During fetal development, as well as in adult life, normal somatic cells (and cancer cells) exhibit alterations in DNA methylation induced by environmental stimuli. As CpGs are paired with GC in the opposite strand, methylation in one strand is mirrored by methylation in the other. During replication, methylation in the parent strands directs methylation in the newly replicated DNA by recruiting DNA methyltransferases.

Table 7.1 A brief overview of epigenetic modifications and their effects on gene regulation

Epigenetic modification	Main outcome and effect
1-DNA methylation	• Modulates gene transcription • Methylation of CpG islands of promoter region is mostly associated with silencing of gene expression • Methylation of CpG dinucleotides located in the gene coding sequence is associated weakly with gene silencing but the opposite
Methylation is rare in CpG-rich regions or CpG islands	
Regions are usually longer than 500 bp	
GC base content > 55 %	
Located in the *promoter regions and* at the end of the 5′ region. Types of promoters: rich and poor in CpG islands	
Methylated sites are distributed globally on about 80 % of CpGs	
Enzymes involved in this process: DNA methyltransferases (DNMTs) DNMT1, DNMT3a, DNMT3b, DNMT2, and accessory proteins, such as DNMT3L	
Stable in somatic cells but modifiable by environmental factors	
Methylation levels may show interindividual heterogeneity	
Different tissues are able to show local differences in DNA methylation	
Non-CpG methylation	• Potentially inducible by environmental factors
Location in the promoter remains controversial	
The functional significance of non-CpG methylation in early development is uncertain	
May be modulated by DNMT3 activity	
Can occur in CpA, CpC, and CpT nucleotides situated in the DNA of embryonic stem cells and episomal DNA	
2-Histone posttranslational modifications	• Implicated in the de novo methylation of DNA • Histone acetylation: associated with more open chromatin and transcriptional activation • Histone hypoacetylation: Associated with condensed chromatin structure and repression of gene transcription
Modifications: – Acetylation, methylation, ubiquitination, and SUMOylation of lysine residues – Phosphorylation of serine residues – Methylation of arginines – Most frequent histone lysine modifications [71]: methylation of histone H3 at Lys9 (H3-K9) or Lys27 (H3-K27) is associated with gene silencing – Methylation or acetylation of histone H3 at Lys4 (H3–K4) or acetylation of H3 at Lys27 (H3-K27) is associated transcriptional activation	
Enzymes involved in these processes: – Histone acetyltransferases (HATs) – Histone deacetylases (HDACs) – Histone methyltransferases – Methyl-binding domain protein MECP2	• HATs can be divided into several families, including the PCAF/Gcn5, p300/CBP, MYST, SRC, TAFII250, HAT1, and ATF-2 families • HDACs are classified into four groups (I–IV)

Subsequently, stable transfer of gene methylation patterns to progeny lines is accomplished. CpG methylation is thought to constrain expansive regions of the genome by silencing repetitive sequences or repressing promoters by recruiting methyl-CpG-binding proteins (the MBD protein family). Although methylation is associated frequently but not always, with repressed promoters, transcriptional repression via histone methylation and/or deacetylation precedes DNA methylation. Table 7.1 gives a short overview of the main features of epigenetic modifications.

The transcriptional coactivator, peroxisome proliferative activated receptor gamma coactivator 1 alpha (*PPARGC1A*), coordinates the regulation of genes involved in energy metabolism by controlling transcriptional programs of mitochondrial biogenesis, adaptive thermogenesis,

and fatty acid beta-oxidation [64]. In fact, its tissue specificity pattern of expression is located mainly in the heart, skeletal muscle, liver, and kidney. Interestingly, the protein encoded by this gene is involved in controlling blood pressure, regulating cellular cholesterol homoeostasis, and in the development of obesity, and altered signaling of *PPARGC1A* contributes to glucose intolerance, insulin resistance, and type 2 diabetes [64, 65]. We focused on methylation of 5-methylcytosine in dinucleotide CpG, which is associated generally with gene silencing, and measured the level of DNA methylation of three putative methylation target sites in the promoter of the *PPARGC1A* (located at positions -513, -519, and -615 relative to transcriptional start site) [66]. Interestingly, we demonstrated that the methylation status of the *PPARGC1A* promoter in the liver of patients with NAFLD is significantly associated with plasma fasting insulin levels and homeostasis model assessment of IR (HOMA-IR), regardless of the liver disease severity [66]. As expected, we observed that the methylation status of the *PPARGC1A* promoter was significantly associated with fatty liver as a disease trait, showing that a higher proportion of the alleles was methylated in NAFLD patients compared with in the liver of control subjects [66]. We also observed that liver *PPARGC1A* mRNA abundance was inversely correlated with the methylation levels of *PPARGC1A* promoter CpGs, suggesting that the methylation of at least the three explored sites in the promoter repressed the transcriptional gene activity efficiently [66].

An interesting study explored the pre- and post-bariatric changes in the methylation profile of nine genes coding for enzymes that regulate intermediate metabolism and insulin signaling in the liver of morbidly obese patients with NAFLD [67]. The most remarkable finding of this study is that therapeutic intervention partially reverted NAFLD-associated methylation changes; for instance, the gene encoding protein-tyrosine phosphatase epsilon (*PTPRE*) showed both differential expression and differential methylation before and after bariatric surgery [67]. Moreover, the authors observed that the insulin-like growth factor-binding protein 2 (*IGFBP2*) locus was hypermethylated and its mRNA downregulated in NASH.

Murphy and colleagues, who recently did global methylation profiling of liver samples of NAFLD patients at different stages of disease severity by using the Illumina HumanMethylation450 BeadChip platform, observed that patients with advanced NAFLD had a signature of differentially methylated CpG sites that allow discrimination between advanced versus mild disease [68]. Indeed, the authors showed that advanced NAFLD has a relative hypomethylation state (11 % of 52,830 CpG sites) compared with mild NAFLD, specifically in genes associated with tissue repair, for instance, *FGFR2* (a fibroblast growth factor receptor family member), genes of the collagen (*COL1A1*, *COL1A2*, *COL4A1*, and *COL4A2*) and laminin families, and many chemokines [68]. Of note, genes involved in pathways that generate methyl groups, including methylenetetrahydrofolate dehydrogenase 2 (*MTHFD2*), were significantly hypomethylated in advanced NAFLD [68].

Finally, we recently described a novel disease mechanism associated with NAFLD progression that involves epigenetic changes of mitochondrial DNA (mtDNA) [69]. In our study, we explored the status of cytosine methylation of liver mtDNA in target regions of the mtDNA genome for the first time. We observed that the methylation levels of mitochondrial NADH dehydrogenase 6 (MT-ND6), the gene that encodes for a key enzyme of complex 1 of the o*xidative-phosphorylation (OXPHOS) chain,* were higher in the livers of NASH patients and that there was a clear decrease in the protein levels and changes in mitochondrial morphology, suggesting that the methylation status of this mitochondrial gene plays a role in the phenotypic switching from SS to NASH [69]. To contrast with the hypothesis that epigenetic modifications might be reversible by intervention, we explored whether the observed changes were associated with interventional programs, observing that physical activity modulates the methylation status of MT-ND6 [69].

On the other hand, it is known that chronic ethanol intake lowers the hepatocellular

S-adenosylmethionine-to-S-adenosylhomocysteine ratio and significantly impairs liver methylation reactions [70]. Recently, Kharbanda and colleagues showed that chronic alcohol consumption is associated with a decrease in the hepatic methylation capacity, causing liver injury. Of note, the authors suggested that dietary intervention might be recommended to subjects with AFLD, as exposure to methyl-group consumers, such as guanidinoacetate, can aggravate the liver toxicity of ethanol [70].

Furthermore, it was shown that ethanol increases acetylation of H3-Lys9 through modulation of HAT(s) [71]. A comprehensive review on ethanol and epigenetic modifications was published recently [72].

In summary, epigenetic modifications emerged as an interesting target of therapeutic intervention in chronic and prevalent human diseases as they offer a unique framework of reversible mechanisms that modulate cellular transcriptional machinery.

The Role of Mitochondrial Dysfunction in the Development and Disease Progression of NAFLD and AFLD

Resembling our observations about the critical role of *PPARGC1A* on the development of NAFLD, in experimental models, Lieber and coworkers demonstrated that alcohol reduces the hepatic expression of *PPARGC1A* mRNA by about 50 % [73], reinforcing the importance of master regulators of metabolism in the energy sensing system of the liver that also lead to mitochondrial dysfunction and damage [66, 69, 73]. In agreement with these observations, Han et al. reported that liver mitochondria undergo dynamic alterations following chronic alcohol feeding in mice that include increased mitochondrial NAD^+ and NADH levels with enhanced mitochondrial biogenesis in the liver to adapt to metabolic stress [74]. Our group observed a similar finding in rats, demonstrating that in rodents, metabolic insults, like high-fat diet, promote an increase in liver mitochondrial biogenesis in response to hypoxia via HIF-1alpha, probably to enhance the mitochondrial function and to accommodate the metabolic stress [75]. Nevertheless, once the NAFLD is established in humans and the disease progresses to NASH, mitochondrial DNA content is significantly reduced in the liver, suggesting that the advanced disease is associated with a loss of the adaptive mechanism for the metabolic demands [66].

miRNAs in NAFLD and AFLD Biology

The regulation by microRNAs (miRNAs) of transcriptional and posttranscriptional gene expression and mRNAs translation to proteins is perhaps one of the most exciting mechanisms affecting human disease biology because of the dramatic influence they can exert on their target gene expression by acting on mRNA stability and translation and/or chromatin structure modifications. They also emerged as potential biomarkers of disease severity and a novel autocrine, paracrine, and even endocrine system [76].

As highlighted previously, fatty acid metabolism (FAM) is deregulated in both NAFLD and AFLD; thus we explored a validated gene-miRNA interaction search in FAM-KEGG pathways using the bioinformatic resource miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/pathwaytarget.html); Table 7.2 shows the results. Notably, the interaction network is complex, and miR-373, miR-33, miR-34, miR-155, miR-122, and miR-124 seem to be highly represented.

Moreover, an integrative functional analysis performed by our group showed novel associations with miRNAs that might modulate AFLD, such as miR-9, or NALFD, such as miR-146a, miR-18a, and miR-22 [4], and/or both (miR-7a and miR-199a-3p). These miRNAs were predicted as being related to the apoptosis- and inflammation-related network integrated by IL6, BCL2, caspase 3, NF-κ-β, CD40L, MAPKs, and PTEN [4].

Of particular interest are miR-155 induced both in vitro and in vivo by alcohol (Kupffer cells), contributing to increased TNF alpha production [77], and miR-122 that plays a remarkable role in

Table 7.2 Prediction of validated gene-miRNA interaction search in the KEGG fatty acid metabolism pathway

Gene name	Entrez ID	MicroRNA name
EHHADH	1962	hsa-miR-532-5p
HADH	3032	hsa-miR-373
HADHB	10449	hsa-miR-373
ALDH9A1	3033	hsa-miR-373
ACSL1	223	hsa-miR-373
ACSL1	10455	hsa-miR-373
HADHB	3032	hsa-miR-373
CPT1A	10449	hsa-miR-373
HADHB	3033	hsa-miR-373
CPT1A	223	hsa-miR-373
HADH	10455	hsa-miR-373
ALDH1A3	1374	hsa-miR-370
HADH	2180	hsa-miR-34c-5p
PECI	2180	hsa-miR-34c-3p
PECI	2180	hsa-miR-34b
HADHB	2180	hsa-miR-34b
HADH	2180	hsa-miR-34a
CPT1A	2180	hsa-miR-34a
HADH	3032	hsa-miR-33b
ALDH9A1	1374	hsa-miR-33b
ACAA2	3032	hsa-miR-33b
ALDH9A1	1374	hsa-miR-33b
ALDH1A3	3032	hsa-miR-33a
EHHADH	1374	hsa-miR-33a
HADH	3032	hsa-miR-33a
HADHB	1374	hsa-miR-33a
ALDH9A1	3032	hsa-miR-33a
ACSL1	1374	hsa-miR-33a
ACSL1	3032	hsa-miR-33a
HADHB	1374	hsa-miR-33a
CPT1A	3033	hsa-miR-21
HADHB	3033	hsa-miR-21
CPT1A	220	hsa-miR-181c
HADH	220	hsa-miR-181c
ALDH1A3	501	hsa-miR-155
HADH	501	hsa-miR-155
PECI	3033	hsa-miR-124
PECI	223	hsa-miR-124
HADHB	223	hsa-miR-124
HADH	10455	hsa-miR-124
CPT1A	10455	hsa-miR-124
HADH	3032	hsa-miR-124
ALDH9A1	10455	hsa-miR-124
ACAA2	3032	hsa-miR-124
ALDH9A1	10449	hsa-miR-124
ALDH1A3	3032	hsa-miR-124
EHHADH	3033	hsa-miR-124
HADH	10449	hsa-miR-124
HADHB	3033	hsa-miR-124
ALDH9A1	10449	hsa-miR-124
ACSL1	223	hsa-miR-124
ACSL1	1374	hsa-miR-122
HADHB	1374	hsa-miR-122
CPT1A	3033	hsa-miR-10b
HADHB	3033	hsa-miR-10b
CPT1A	10449	hsa-miR-1
HADH	3033	hsa-miR-1
ALDH1A3	223	hsa-miR-1
HADH	10455	hsa-miR-1
PECI	3032	hsa-miR-1
PECI	10449	hsa-miR-1
HADHB	10455	hsa-miR-1
HADH	3033	hsa-miR-1
CPT1A	223	hsa-miR-1

Prediction was performed by miRWalk program at http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/pathwaytarget.html

NAFLD biology and alanine aminotransferase regulation [78]. The interactome predicts other targets, such as TGF-β, shown to be deregulated in liver biopsies and platelets from NAFLD patients [79]. Table 7.3 summarizes novel findings on miRNAs in the biology of human NAFLD and ALD.

To summarize, the body of evidence from genetics, epigenetics, and pathophysiological studies indicates that despite NAFLD and AFLD having specific characteristics, such as the environmental *noxa*, they share many common deregulated metabolic pathways and probably similar underlying susceptibilities [4]. It is worthy to note that recent evidence may indicate that a modest alcohol intake may be beneficial in NAFLD patients [80].

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Table 7.3 Evidence from human studies regarding the role of miRNAs in the biology of NAFLD and AFLD

miRNA	Disease model	Main finding	References
Circulating and liver expression miR122	Patients with NAFLD and controls diagnosed by liver biopsy	Circulating miR-122, miR-192, miR-19a and 19b, miR-125b, and miR-375 were upregulated > twofold in simple steatosis or nonalcoholic steatohepatitis	[78]
		In NAFLD patients, the majority of serum miR-122 circulates in argonaute2-free forms	[76]
		Liver miR-122 expression is tenfold downregulated in NASH compared with simple	[76]
		Liver miR-122 expression is expressed preferentially at the edge of lipid-laden hepatocytes	[76]
		miR-122 might interact with *GPT1* at multiple sites of the coding region to enhance translation	[76]
		A functional variant (rs41318021) in the 3′ UTR region of a validated miR-122 target gene involved in endothelial damage (*SLC7A1*) is significantly associated with arterial hypertension	[76]
		The silencing of miR-122 is an early event during hepatocarcinogenesis from NASH	[81]
Circulating miR-19a/b and miR-125b	Patients with NAFLD and controls diagnosed by liver biopsy	Circulating miR-19a/b and miR-125b correlate with biomarkers of atherosclerosis	[78]
Circulating miR-21, miR-34, miR-122, and miR-451	Patients with NAFLD, intrahepatic fat assessed by ultrasound scan	Circulating levels of miR-21, miR-34a, miR-122, and miR-451 are overexpressed in NAFLD The serum level of miR-122 correlated with the severity of liver steatosis	[82]
Circulating miR-15b	Patients with NAFLD and controls assessed by ultrasound	miR-15b is upregulated in the serum of fatty liver disease patients compared with healthy subjects	[83]
Liver expression miR-34a	Liver biopsies were obtained from NAFLD morbid obese patients undergoing bariatric surgery	miR-34a, apoptosis, and acetylated p53 increased with disease severity	[84]
Liver expression miR-34a	Obese patients with NAFLD and controls diagnosed by liver biopsy	miR-34a, a microRNA increased in NAFLD, inhibits sirtuin-1 with downstream dephosphorylation of AMP kinase and HMGCR	[85]
Visceral adipose tissue (VAT) miR-197 and miR-99	Visceral adipose tissue samples were collected from NAFLD patients	miRNA expression from VAT may contribute to the pathogenesis of NAFLD	[86]
Circulating miR-34a and miR-122-	Patients with NAFLD proven by liver biopsy, no controls	miR-34a and miR-122 represent noninvasive biomarkers of diagnosis and histologic disease severity	[87]
Liver expression miR-122 and miR-34a	Patients with NAFLD proven by liver biopsy and controls	miR-122 level is significantly decreased in the liver of subjects with NASH miR-34a and miR-146b levels were significantly increased in subjects with NASH	[88]
Circulating miR-214	Patients with alcoholic steatohepatitis	Circulating levels of miR-214 is proposed for noninvasive diagnosis of ASH	[89]

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8 Clinical Features, Disease Modifiers, and Natural History of Alcoholic Liver Disease

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Introduction

Alcoholic liver disease (ALD) is a major cause of morbidity and mortality worldwide [1]. The clinical spectrum of ALD includes fatty liver, steatohepatitis with or without fibrosis, and cirrhosis. Alcoholic hepatitis (AH) is an important cause of morbidity, mortality, and health-care costs in the USA and worldwide. In 2007, 56,809 patients (0.71 % of the total) were hospitalized in the USA with the ICD-9 diagnosis of AH [2]. Average length of stay was 6.5 days, and average hospital costs were $37,769, which is more than twice the cost of myocardial infarction and approximately four times the cost of acute pancreatitis. A nationwide study on AH in Denmark from 1999 to 2008 [3] found that over that time period the 28-day mortality rose from 12 to 15 % and the 84-day mortality from 14 to 24 %. The overall 5-year mortality was 56 %, 47 % in those without cirrhosis, and 69 % in those with cirrhosis. These data from Denmark are quite similar to VA Cooperative Studies data on 4-year mortality: 42 % mortality with AH alone and 65 % with AH plus cirrhosis [4]. Thus, despite increases in knowledge of mechanisms for AH, mortality is not improving for this important clinical problem.

The diagnosis of ALD is made in patients with evidence of liver injury based on clinical history, physical findings, and laboratory abnormalities, when there is evidence of significant alcohol consumption and after other causes of chronic liver disease have been excluded. Problems

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diagnosing the disease arise when the patient exhibits no symptoms or clinical findings to suggest the diagnosis or when the patient conceals alcohol abuse. The situation is even more difficult when the patient has risk factors for other causes of liver disease, such as obesity or diabetes mellitus, or has superimposed viral hepatitis. The goal of this chapter is to review the clinical features that support the diagnosis, selected disease modifiers, and natural history of ALD.

Clinical Features of Alcoholic Liver Disease

Clinical History

To obtain the history of hazardous alcohol consumption, the US Preventive Services Task Force recommends annual routine screenings of all adults, followed by brief counseling if the result is positive [5]. The most commonly employed validation tool to detect hazardous alcohol consumption is the Alcohol Use Disorders Identification Test (AUDIT). A score of 8 or more (7 or more for adults over age 65) indicates alcohol use disorder or alcohol dependence (sensitivity >90 % and specificity >80 %). A shorter screening can be done with the 3-question AUDIT-C tool that gives zero to 4 points for the answer to each question and is considered positive for males with a score of ≥4 points and for females with a score of ≥3 points [6], with moderate risk being 3–5 points, high risk 6–7 points, and severe risk 8–12 points.

The National Institute on Alcohol Abuse and Alcoholism has a single-question test: "How many times in the past year have you had 5 or more drinks for males, or 4 or more drinks for females, in a day?" An answer of one or more times constitutes a positive test. This question has a sensitivity of 82 % and specificity of 79 % for unhealthy alcohol use [7]. A less powerful tool is the CAGE questionnaire (the name is an acronym of its four questions) in which two or more positive answers indicate hazardous alcohol use [8].

Depending on individual susceptibility, alcohol-induced organ injury requires alcohol consumption of ≥20 g per day for females or ≥40 g per day for males; however, larger amounts than this threshold are usually needed. More than 60 % of individuals who drink more than 60 g of alcohol a day will develop fatty liver [9, 10]. A "standard" alcohol drink has 14 g of alcohol and is equivalent to 12 oz. of beer, 5 oz. of wine, 8–9 oz. of malt liquor, or 1.5 oz. of distilled spirits (whiskey, bourbon, etc.). The value of the alcohol intake history depends on the recall ability and the truthfulness of the patient.

Symptoms

The symptoms of ALD vary from nonexistent to severe and life threatening (Table 8.1). Most patients with alcoholic steatosis are asymptomatic, but some may have nonspecific symptoms such as abdominal fullness or fatigue. Patients with alcoholic hepatitis frequently have abdominal fullness (up to 80–90 % of cases), jaundice

Table 8.1 Signs and symptoms in alcoholic hepatitis/cirrhosis

Physical exam findings		Symptoms
RUQ tenderness 2+	Confusion 1+	RUQ abdominal discomfort 2+
Hepatomegaly 2+	Fever 1+	Weight loss/gain 2+
Ascites 2+	Finger clubbing 1+	Anorexia 2+
Malnutrition and wasting 2+	Dupuytren's contractures 1+	Fatigue 2+
Venous collaterals 1+	Leg edema 1+	Muscle cramps 2+
Splenomegaly 1+	Parotid gland enlargement 1+	Pruritus 1+
Jaundice 1+	Gynecomastia 1+	Nausea and vomiting 1+
Palmar erythema 1+	Testicular atrophy 1+	Confusion/mental disturbance 1+
Cutaneous telangiectasia 1+	Dementia 1+	GI bleeding 1+
Asterixis 1+	Peripheral neuropathy 1+	Sleep disturbance 1+

(37–60 %), fever (23–56 %), abdominal distention (35–57 %), gastrointestinal bleeding (10–23 %), changes in consciousness (18–45 %), and abdominal pain [11, 12].

Patients with compensated cirrhosis are frequently asymptomatic, but they may have anorexia, nausea, weight loss, fatigue, weakness, abnormal menstruation, loss of libido, muscular cramps, and/or difficulty concentrating on mental tasks. Patients with decompensated cirrhosis are often jaundiced, have evidence of muscular wasting, feel weak, and develop fluid retention with edema and abdominal distention. In addition, many complain of itching and others present with hematemesis or melena. Easy bruising, inverted sleep pattern, and confusion are also frequent complaints.

Physical Exam

On physical exam, signs of fatty liver can range from mild hepatomegaly, with blunting of the normally sharp liver edge, to massive hepatomegaly (Table 8.1). In patients with alcoholic hepatitis, hepatomegaly (80–90 % of cases), jaundice (40–60 %), and fever (23–56 %) are very common. If the injury is severe, the patients will have ascites, hepatic encephalopathy, splenomegaly, evidence of muscle wasting, and sometimes gastrointestinal hemorrhage with portal hypertensive gastropathy or gastroesophageal varices [11, 12].

Patients with compensated cirrhosis often have hepatomegaly with hard liver consistency and a nodular surface. They may also have splenomegaly and, less often, right upper quadrant pain. Less common findings include gynecomastia and testicular atrophy [13], amenorrhea, parotid enlargement [14], cutaneous spider angioma [15], Dupuytren's contractures [16], digital clubbing [17] (found especially in patients with hypoxemia related to hepatopulmonary syndrome), palmar erythema, and nail changes.

Patients with decompensated cirrhosis may also have mental changes of hepatic encephalopathy with variable degrees of confusion, with or without asterixis, jaundice, ascites, and peripheral edema. Some patients will have evidence of gastrointestinal bleeding or other organ damages, including alcoholic gastritis or pancreatitis, alcoholic neuropathy, or, less commonly, alcoholic cardiomyopathy. Signs of alcohol withdrawal are common in patients with alcoholic hepatitis who have recently discontinued alcohol.

Laboratory

Patients with ALD should have a complete blood count, international normalization ratio (INR), comprehensive metabolic panel, and GGT. Because there is the risk of overlapping viral hepatitis, serologies for current infection or past exposure to hepatitis A, B, and C are advised. Patients who are not immune should be vaccinated against hepatitis A and B. If the ALT is elevated, a full workup for other causes of liver disease and cirrhosis is also advisable because other immune or metabolic causes may be uncovered.

Common hematologic findings include anemia, macrocytosis, leukopenia, lymphocytopenia, and thrombocytopenia. Macrocytosis is often due to alcohol toxicity to the bone marrow, but is important to assess for folic acid and/or vitamin B12 deficiency. In patients with alcoholic hepatitis without cirrhosis, thrombocytopenia frequently reverses with rebound thrombocytosis after 1–3 weeks [18]. In cirrhotic patients, thrombocytopenia persists as evidence of portal hypertension. An elevated INR is a marker of the severity of the disease, but can also signal a nutritional deficiency; hence, vitamin K repletion can be helpful in clarifying the situation by eliminating the nutritional deficiency component.

The comprehensive metabolic panel helps to identify electrolyte imbalances that need prompt correction. Similarly, measurements of magnesium, zinc, and phosphorous in plasma can be useful. We frequently supplement with magnesium (magnesium oxide 400 mg/day) and zinc (zinc sulfate 220 mg/day) to treat muscle cramps. Patients with alcoholic hepatitis and with decompensated cirrhosis are very susceptible to kidney injury, and creatinine elevated above the patient's usual "baseline" requires prompt attention and intervention to avoid progression of acute kidney injury and development of hepatorenal syndrome.

Consumption of alcohol in excess of 50 g per day causes elevation of AST (sensitivity 50 %, specificity 82 %) and ALT (sensitivity 35 %, specificity 86 %) [19]. The elevation of AST is typically higher than that of ALT, and 79 % of patients with alcoholic hepatitis have an AST–ALT ratio >2. Patients with alcoholic hepatitis with ALT > AST frequently have overlapping causes, such as superimposed viral hepatitis or drug injury (most often acetaminophen); the same is true if the AST or ALT is higher than 300 units/L, because this threshold is not likely to be surpassed by alcohol injury alone. Elevated GGT is more sensitive for alcohol abuse (56–73 %) but less specific (53–70 %) than CDT or MCV [20]. Using a combination of these tests may be warranted. Elevated bilirubin, in the absence of biliary obstruction, is a marker of the severity of the alcoholic liver injury and is very important as a component of the Maddrey discriminant function, MELD score, Glasgow alcoholic hepatitis score, the Lille model, and the Child–Turcotte–Pugh calculator index (discussed subsequently).

Validated self-reported questionnaires are superior to biochemical tests in detecting alcohol abuse. Biochemical tests can be utilized in situations where the suspicion of alcohol abuse is high but the patient denies alcohol abuse. Carbohydrate-deficient transferrin (CDT) is elevated in alcohol abuse but is a less useful marker in females [21, 22] and in patients with cirrhosis [23]. Combinations of CDT, MCV, and/or GGT can help improve sensitivity and specificity [24–26] for heavy alcohol consumption.

Imaging

For patients with alcohol abuse who have significant fat in the liver (≥30 % fat), ultrasound will detect diffuse hyperechoic texture, with a sensitivity of 91 % and a specificity of 93 %. The sensitivity is only 64 % with fatty infiltration less than 30 % [27]. CT scan without contrast is highly predictive of fatty liver when the liver-to-spleen attenuation ratio is more than ten Hounsfield units [28]. MRI is the best tool but is more expensive, with a sensitivity of 95 % and specificity of 98 % [29]. When trying to identify advanced fibrosis or cirrhosis, ultrasound has lower sensitivity (50–70 %) with specificity of 88 % [30]. Multiphase CT scan and MRI are superior in identifying cirrhosis and its complications, including collateral circulation, vascular thrombosis, hepatocellular carcinoma, or other focal liver lesions. Ultrasound is the most commonly used initial imaging technique, helpful in identifying biliary dilation in a patient with jaundice and in the detection of ascites.

Liver Biopsy

In the presence of a history of alcohol abuse associated with typical liver enzyme elevations, diagnosis is very reliable with sensitivity of 91 % and specificity of 97 % [31], and liver biopsy is often not performed in the clinical (non-research) setting. Patients with atypical presentation or who have markers of other types of liver disease (autoimmune hepatitis, hemochromatosis, viral hepatitis, etc.) are good candidates for liver biopsy. Liver biopsy helps to clarify the diagnosis and will give the stage of disease, which is useful in deciding if surveillance for hepatocellular carcinoma is needed. Liver biopsy also differentiates simple steatosis from steatohepatitis but is rarely needed for this purpose. The histologic findings of alcoholic steatohepatitis include centrilobular steatosis, hepatocyte ballooning, Mallory bodies, perivenular fibrosis, pericellular fibrosis, and mixed inflammatory infiltration of neutrophils and lymphocytes. Megamitochondria are often seen in cases of recent alcohol abuse. Patients with cirrhosis frequently have micronodular changes and bile duct proliferation. Less often, patients have mixed macro- and micronodular cirrhosis.

Alcoholic Liver Disease Modifiers

Introduction

Many people drink heavily, yet only a limited number (~35 %) develop more advanced liver diseases (alcoholic hepatitis or cirrhosis). Thus, there

Table 8.2 Disease modifiers

Continued drinking
Age, Sex
Race
Diet/Nutrition
Genetics/Epigenetics/Family History
Smoking
Obesity
Occupational/Environmental Exposure
Medications/Drugs of Abuse
Other Liver Diseases

must be modifying factors that either prevent or facilitate disease activity/progression. These modifiers can either be fixed (e.g., genetics) or can undergo intervention (e.g., smoking, diet). We review ten disease modifiers of particular importance to ALD (Table 8.2). Continued drinking, the most compelling modifier, is discussed throughout this chapter.

Gender and Age Differences

Males and females have differences in the absorption, distribution, and metabolism of alcohol [32, 33]. Females have a lower proportion of body water than males of equal body weight; therefore, they can achieve higher concentrations of blood alcohol even with equivalent amounts of alcohol [34]. Some investigations show that the gender variability in peak blood alcohol concentrations following equivalent low doses of alcohol could be due to the differences in first-pass metabolism of alcohol in the gastrointestinal tract, which was significantly correlated with gastric alcohol dehydrogenase (ADH) activity. Gastric ADH activity was lower in females [35, 36]. However, other studies have demonstrated no gender differences in the first-pass metabolism of alcohol [37] and indicated that first-pass metabolism was evident only with the ingestion of relatively low doses of ethanol and when gastric emptying is slow [38].

Epidemiological data also show that females are more susceptible to alcohol-related liver damage than men [39]. Women can develop ALD as alcoholic cirrhosis and alcoholic hepatitis at younger ages, at lower rates of daily alcohol intake, and at lower cumulative exposure to alcohol than the males [9]. Two hypotheses have been put forward to explain this gender-based difference. The first is related to gender-based variability in alcohol pharmacokinetics (PK), and the second is related to gender-based differences in the metabolic processes that are influenced by chronic alcohol use, such as the generation of free radicals, fatty acid metabolism, and endotoxins [40]. The mechanism for the former may be a larger liver volume or enhanced function in males, including the stimulation of alcohol-metabolizing enzymes in the liver, resulting in an alteration in the PK of alcohol. The latter is thought to be related, at least in part, to gender-based differences in portal endotoxemia, hepatic inflammatory responses, and the activation of Kupffer cells [41–43]. Both mechanisms may be related to fundamental differences between women and men in sex steroids, including estrogen, and their influence on alcohol metabolism as well as on liver disease susceptibility.

The role of age–gender interactions in response to the effects of alcohol is of interest, although there has been limited research in this area. The elderly are thought to be more sensitive to alcohol and show greater impairment than younger groups. However, it is not clear if these changes are due to pharmacokinetic or pharmacodynamic factors [44]. Pharmacokinetic changes, including a decrease in circulation volume, can result in increased alcohol levels and therefore increased impairment, in older participants following standard doses of alcohol. Age is a predictor for ALD prognosis [45]. Age and gender (and their potential interaction) also have been reported in various stages of ALD as risk factors that were associated with disease severity [46].

Race

Data are relatively consistent concerning race/ethnicity and ALD. Research from large multicenter Veterans Affairs Cooperative Studies showed that alcoholic cirrhosis was more frequent in Hispanics (73 %) than in non-Hispanic

White/Caucasians (52 %) and African-Americans (44 %) with acute alcoholic hepatitis. African-Americans also were more likely to have hepatitis C as a confounder. Data from the Fourth National Health and Nutrition Examination Survey (NHANES IV) was used to evaluate ethnic differences and AST/ALT values. Mexican-American men and women were the most likely to have elevated aminotransferase activity. Among men, Mexican-Americans were more likely than whites to be heavy/binge drinkers. African-Americans have consistently shown to be more likely to have hepatitis B or hepatitis C [47]. Recent studies at the University of California Davis Medical Center showed that Hispanic patients presented 4–10 years earlier than their White/Caucasian counterparts in all states of severity of ALD [48]. There were more obese Hispanic patients than White/Caucasian patients.

Diet/Nutrition

Diet and nutrition play a major role in ALD, and patients with ALD show various degrees of nutritional deficiency [49]. Malnutrition is a multifactorial consequence of ALD [50] and has been best studied in patients with alcoholic hepatitis (AH) (which has a high association with malnutrition [51]). The most comprehensive reports on malnutrition in ALD have come from large studies conducted by the Veterans Health Administration (VA) Cooperative Studies Program in patients having AH [51–54]. Almost every AH patient showed some degree of malnutrition [52]. Further, approximately 50 % of patients' energy intake came from alcohol. Although calorie intake was frequently not inadequate, intake of protein and critical micronutrients was often deficient. Importantly, the severity of liver disease and mortality correlated the severity of malnutrition (Fig. 8.1). Patients were given a balanced 2500-kcal hospital diet and encouraged to consume the diet. Voluntary oral food intake correlated in a stepwise fashion with 6-month mortality data. Thus, patients who voluntarily consumed more than 3000 kcal/day had virtually no mortality, whereas those consuming less than 1000 kcal/day had greater than 80 % 6-month mortality [51] (Fig. 8.1). Importantly, anorexia was a frequent finding in these AH patients, and it correlated with severity of liver disease. Moreover, alcohol consumption itself can also lower food craving [55].

Dietary fat represents a macronutrient dietary modifier for ALD. Many investigations have shown that dietary saturated fat protects against alcohol-induced liver disease in preclinical models using rodents, whereas dietary unsaturated fat, enriched in linoleic acid (LA) in particular, promotes alcohol-induced liver damage [56–58]. LA is enzymatically converted to bioactive oxidation products, OXLAMs, primarily via the actions of 12/15-lipoxygenase (12/15-LO) or nonenzymatically via free radical-mediated oxidation in response to oxidative stress. LA is the most abundant polyunsaturated fatty acid in the human diet [59] and in human plasma and membrane lipids. Dietary intake of LA has more than tripled over the past century. We have shown that an LA-enriched diet enhances intestinal inflammation, endotoxemia, Toll-like receptor activation, and liver injury in a mouse model of ALD [60, 61]. We postulate that the type of dietary fat consumed may explain, at least in part, why only some people who drink heavily develop ALD.

Zinc deficiency is a micronutrient deficiency commonly observed in ALD. Zinc is an essential trace element required for normal cell growth, development, and differentiation. It is a critical component in many zinc proteins/enzymes, including critical zinc transcription factors. Some of the mechanisms for zinc deficiency/altered metabolism include decreased dietary intake, increased urinary excretion, and alterations in certain zinc transporters. Importantly, oxidative stress (through modification of the cysteines that retain zinc) may also cause loss of zinc from critical zinc finger proteins [62]. Zinc deficiency may manifest itself in many ways in liver disease, including skin lesions, poor wound healing, liver regeneration, altered mental status, or altered immune function. Zinc supplementation has been documented to block/attenuate experimental ALD through multiple processes, including stabilizing gut-barrier function, decreasing endotoxemia, decreasing proinflammatory cytokine production, decreasing oxidative stress, and

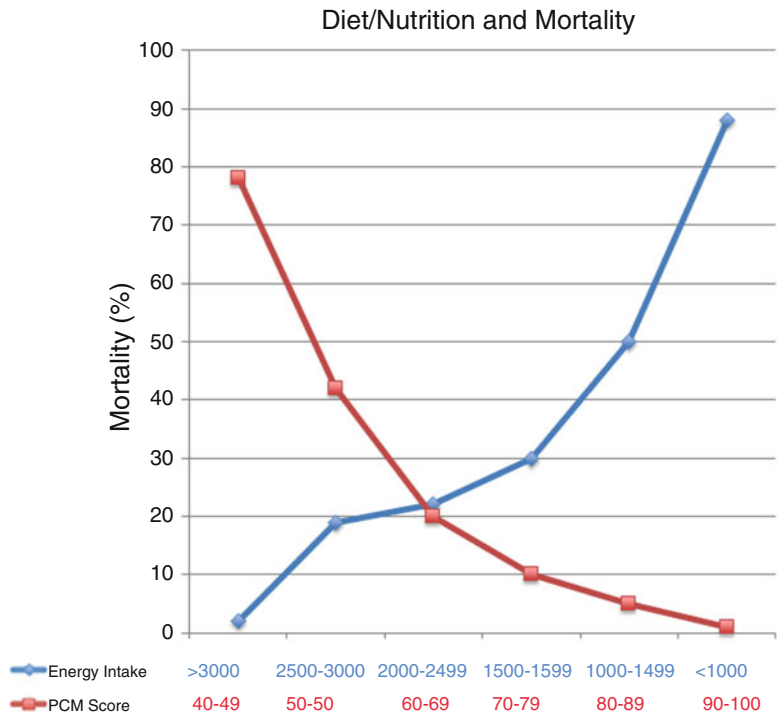

Fig. 8.1 Data from VA Cooperative Studies demonstrate a dose–response relationship between nutrition and outcome. As shown in *red*, the protein–calorie malnutrition (PCN score) decreased in an inverse, indirect relation with mortality. A normal score is 90–100, and the lower the score, the more severe the malnutrition. Similarly, voluntary calorie intake directly correlated with mortality (shown in *blue*). Those who ate ≤1000 cal had >90 % mortality, while those who ate >3000 cal had virtually no deaths

attenuating apoptotic hepatocyte death [62, 63]. Clinical trials in human liver disease are limited in size and quality, but it is clear that zinc supplementation reverses clinical signs of zinc deficiency in patients with ALD (some studies suggest improvement in liver function in both ALD and hepatitis C following zinc supplementation). The dose of zinc used for treatment of liver disease is usually 50 mg of elemental zinc taken with a meal to decrease the potential side effect of nausea.

Smoking

Nicotine dependence is common among people with alcohol use disorders (AUD), and the amount of smoking directly correlates with level of alcohol consumption and the severity of alcohol problems. Alcohol consumers, including heavy users or those with an AUD, are more likely to smoke cigarettes and be nicotine dependent, and the level of nicotine dependence/smoking correlates directly with levels of alcohol consumption [64]. Subjects with AUD are also more likely to have started smoking earlier in adolescence than smokers without an AUD [65, 66]. There may be common genetic factors in both smoking and alcohol use behaviors [67, 68].

Animal studies have established the role of nicotinic receptors in alcohol reward and provide strong evidence that alcohol and nicotine may act on the same brain pathways—particularly the mesolimbic dopamine system—to exert their rewarding effects and modulate consumption [69, 70]. Furthermore, nicotine administration has been found to increase alcohol consumption, particularly in men [71]. Nicotine is more reinforcing in alcohol-dependent people than in those who have never been dependent, and it increases alcohol consumption in male smokers who drink below hazardous levels.

Cigarette smoking can adversely impact certain hepatic functions and has been associated with severity in ALD in humans. In preclinical studies, cigarette smoking has been shown to have adverse effects on cytochrome P450 and UDP-glucuronosyltransferase activity in the liver [72]. Cigarette smoking can induce oxidative stress (which plays a mechanistic role in ALD). Further, nicotine-derived nitrosamine ketone (NNK) has been shown to have major role in the

pathogenesis of steatohepatitis in a "chronic-plus-binge" rat model of alcoholic liver disease [73]. Cigarette smoking has also been shown to be a risk factor in the development of human alcoholic cirrhosis, especially in those smoking one or more packs per day [74]. Active tobacco use is a significant independent predictor of mortality ($p = 0.03$) [75].

BMI, Excess Weight, and Obesity

The obesity epidemic poses serious and multifaceted health problems in the USA, and recent data from 2011 to 2012 has shown that approximately 35 % of adults and 17 % of children and adolescents are affected by obesity [76]. Significant differences exist by ethnicity, age, and gender, with respect to the prevalence of overweight/obesity [77]. Excess weight and obesity have been known for decades to have negative consequences to liver health [78], and overweight/obesity has been considered as a risk factor for multiple diseases [79]. Obesity has been shown to have a close association with various forms and stages of ALD [46]. Overweight patients with no alcohol drinking history may have nonalcoholic fatty liver disease (NAFLD) with or without fibrosis [80], and/or cirrhosis, and, potentially, hepatocellular carcinoma [81]. Excessive fat in humans and elevated free fatty acids in patients with ALD could contribute to liver injury [82]. Body mass index (BMI) and fibrosis of the liver are positively correlated, and BMI is an independent risk factor for fibrosis in ALD [83]. The presence of overweight/obesity for at least 10 years was independently correlated with the presence of cirrhosis [46]. Excess body weight in patients with heavy alcohol consumption could markedly increase the severity of steatosis and is a risk factor for the development of more advanced stages of ALD, namely, acute alcoholic hepatitis and cirrhosis.

Genetics/Epigenetics/Family History

Recent studies have shown that both genetic and epigenetic factors are important for disease pathogenesis and progression in alcoholic liver disease (ALD). The genetic variations are often associated with conformational changes in protein structures and functions due to single nucleotide polymorphisms (SNPs). Genome-wide association studies (GWAS) have identified around 3.1 million SNPs that can contribute to disease states, and these SNPs may increase or decrease the function of encoded proteins. In ALD, polymorphisms of alcohol-metabolizing enzymes such as ADH and CYP2E1 as well as antioxidant enzymes and cytokine coding genes have shown strong correlation with the progression of ALD [84].

Epigenetic changes, including microRNAs, DNA methylation, and histone modifications, occurring in response to alcohol are known to produce diverse organ/tissue-specific effects. The role of miRNA is well recognized in ALD; some miRNAs play a causal role in disease development, whereas others may be mere associations. A decrease in miR-122 and induction in miR-155 expression has been reported in models of ALD; miR-122-deficient mice develop greater steatohepatitis and fibrosis, while TNF and CEBP are the targets of miR-155. Alcohol-induced alterations in miRNAs are associated with steatohepatitis [85] and fibrosis [86]. Chronic alcohol exposure also alters miRNAs that affect intestinal permeability during ALD [87]. For example, alcohol induces miR-212 which downregulates tight junction protein-1; also, miRNA-212 is higher in colon biopsies of patients with ALD. Additionally, other microRNAs such as miR-320, miR-486, miR-705, miR-1224, miR-27b, miR-214, miR-199a, miR-192, and miR-183 likely contribute to ALD [85].

DNA methylation of cytosine at C5 at CpG dinucleotide in promoter CpG islands silences transcription, whereas lack of methylation activates transcription. Studies in ALD show that the alcohol-induced decrease in s-adenosyl-l-methionine (SAM, the primary cellular methyl donor) can greatly influence DNA methylation and induce global as well as gene-specific changes leading to altered phenotype. Chronic alcohol consumption is reported to induce global DNA hypomethylation in the liver [88], whereas in peripheral blood cells, hypermethylation of DNA is observed after alcohol consumption in

humans [89, 90]. In the context of hepatocellular carcinoma, aberrant DNA methylation was associated with alcohol intake and hypomethylation of the O6-methylguanine DNA methyltransferase gene [91].

Lastly, several in vivo and in vitro studies have established the important role of alcohol-induced histone modification in the development of ALD. Histone acetylation is regulated by the relative activities of histone acetyltransferase and histone deacetylase enzymes, which are both altered by alcohol. Reduced expression of SIRT 1, a class III HDAC, has been shown in alcohol-exposed hepatocytes and is known to regulate the lipid metabolism pathway. Our own studies support this notion. We have shown that dysregulation of hepatic HDAC expression plays a major role in the binge alcohol-induced hepatic steatosis and liver injury by affecting lipogenesis and fatty acid β-oxidation [92]. Site selective acetylation of histone H3 at lys 9 (H3AcK9), and not at H3 lys14, lys18, and lys23, has been observed in alcohol-exposed primary rat hepatocytes [93]. Along with histone acetylation, alcohol also alters histone methylation and phosphorylation in vitro in rat hepatocytes [94] and in vivo [95]. Further, different modifications at different sites in the same histone (e.g., lys-4, lys-9, ser-10, ser-28, H3) may occur on nucleosomes located in different chromatin domains [96].

An interplay that exists between the various epigenetic mechanisms can determine downstream chromatin remodeling and gene expression; for example, DNA hypermethylation can trigger histone deacetylation, and lower histone acetylation increases DNA methylation. Detailed studies are needed to better understand the cross talk and hierarchical order in epigenetic mechanisms in ALD and how interventions may positively modify epigenetics.

Occupational/Environmental Exposure

Exposure to potential toxins in either the workplace or environment can cause hepatotoxicity which can be exacerbated by alcohol. Vinyl chloride (VC) represents a potential industrial/workplace exposure whose toxicity may be exacerbated by alcohol. We reported that VC induced histologic steatohepatitis that was indistinguishable from alcohol-induced steatohepatitis, and we termed this toxicant-associated steatohepatitis (TASH) [97]. VC is metabolized in a strikingly similar fashion to ethanol, and this could potentially account for the observed similarities between TASH and AH. Although initial studies suggested that, at low substrate concentrations (below 100 ppm), VC is metabolized by a pathway involving alcohol dehydrogenase, most studies have concentrated on the role of CYP2E1 as the initial catalyst of VC metabolism. At concentrations up to ≈220 ppm, VC is metabolized by CYP2E1, forming the highly reactive genotoxic epoxide, chloroethylene oxide. Chloroethylene oxide either spontaneously or enzymatically is converted to chloroacetaldehyde. In either pathway, chloroacetaldehyde is formed. Thus, both alcohol and VC are metabolized through similar pathways to a toxic aldehyde metabolite. Our preliminary research in experimental animals suggests that co-exposure may be more toxic than either agent alone.

Arsenic represents an environment for exposure that has great potential to exacerbate alcohol-induced liver injury. Alcohol promotes arsenic absorption. Both arsenic and alcohol increase reactive oxygen species, deplete hepatic GSH levels, impair mitochondrial function, and cause alterations in DNA methylation [98]. With environmental exposures, there are usually multiple contaminants rather than just one compound such as arsenic. A recent Canadian study evaluated a cocktail of 22 contaminants thought to be clinically relevant (northern contaminant mixture) and showed that both a high-fat diet and alcohol increased fatty liver and liver injury in exposed mice [99].

In summary, because of similarities in metabolism and mechanisms of liver injury, occupational/environmental exposures will be highly relevant to the development/progression of ALD, and these exposures are often overlooked.

Medications/Drugs of Abuse

Alcohol and other drugs (including prescription medications, over-the-counter agents, and illicit drugs) may interact to cause hepatotoxicity, and this hepatotoxicity sometimes can be misdiagnosed as traditional ALD. Acetaminophen hepatotoxicity is a classic form of liver injury that can be enhanced by chronic alcohol ingestion. Chronic alcoholics can be more susceptible to acetaminophen hepatotoxicity for a variety of reasons. They frequently eat diets containing inadequate protein which may adversely affect hepatic glutathione stores. Alcoholics may develop gastrointestinal problems, such as gastritis or pancreatitis, with nausea, vomiting, and decreased food intake that may decrease hepatic glutathione stores. Fasting also has been shown to induce hepatic drug metabolism (P450) in rats, and ethanol and certain higher chain alcohols are well-documented inducers of cytochrome P450 (P450-2E1). Lastly, chronic alcoholics are frequently deficient in nutrient antioxidants, such as selenium, vitamin E, and zinc, and this may augment oxidant liver injury. Thus, for multiple reasons, chronic alcoholics may be predisposed to acetaminophen liver injury [100]. This form of liver injury clinically presents with very elevated AST and ALT levels (often >1000 IU/mL) that distinguish it from ALD.

Alcohol abuse is common among HIV-infected patients. Alcohol abuse frequently lessens compliance with antiretroviral therapy and can also enhance hepatotoxicity of certain HAART regimens [101]. Alcohol may also interact with illicit drugs such as 3,4-methylenedioxy methamphetamine (MDMA; ecstasy) which is an amphetamine-derivative drug commonly consumed at rave parties along with other drugs including alcohol [102]. Alcohol has been shown to enhance MDMA hepatotoxicity in mice.

Other Liver Diseases

Alcohol abuse may accelerate other liver diseases and other liver disease may accelerate ALD [103]. Hepatitis C is much more prevalent (up to tenfold higher) in alcoholics than in the general population and is greatest in those with more advanced liver disease. Alcohol and hepatitis C are thought to interact to cause accelerated liver disease through multiple different pathways including increased oxidative stress, enhanced viral replication, hepatocyte apoptosis, altered gut-barrier function, immune dysfunction, and alteration in epigenetics (miR-122), to name only a few [104].

Distinguishing between patients with alcoholic liver disease and those with secondary iron overload from hereditary hemochromatosis can sometimes be difficult. Patients with alcoholic cirrhosis can have elevated serum iron and ferritin levels and increased hepatic iron levels suggestive of hereditary hemochromatosis [105]. Moreover, 15–40 % of patients with hereditary hemochromatosis consume more than 80 g of alcohol daily [106]. Performing genetic testing for hereditary hemochromatosis will assist in making the correct diagnosis. There also is the possibility that being a heterozygote for hemochromatosis or some other hereditary diseases, such as α1 antitrypsin deficiency, may accelerate the course of ALD.

Natural History of Alcoholic Liver Disease

The phenotypical manifestation of alcohol consumption in the liver, and general health status of an individual, is determined by a complex interplay of varying elements such as drinking habits (duration and quantity of alcohol consumption), environmental agents, and individual factors, as discussed previously [107]. Chronic alcohol consumption can lead to a spectrum of liver injuries which can occur sequentially, separately, or simultaneously in the same patient [108].

Alcoholic steatosis is the most common and initial manifestation of alcoholic liver disease. This lesion is pathologically characterized by both microvesicular and macrovesicular fat accumulation within the hepatocytes, with minimal inflammatory response or hepatic fibrosis [109]. Patients with steatosis are usually asymptomatic and are often diagnosed incidentally on abdomi-

nal imaging (usually in the form of transabdominal ultrasound or computed tomography (CT) scan). Patients typically have normal to very mild elevations of their liver enzymes, such as gamma-glutamyl transpeptidase (GGT), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Serum bilirubin and other markers of hepatic function (international normalized ratio (INR), albumin levels) also tend to be normal. Alcoholic steatosis is a relatively benign condition which is reversible with abstinence; however, it has the potential to progress to alcoholic hepatitis or cirrhosis in an accelerated fashion in a minority of patients who continue to drink heavily [110].

Early studies by Dr. Charles Lieber in volunteers demonstrated the relative ease with which alcohol consumption causes fatty liver [111, 112]. In the first study, five volunteer subjects with a history of alcoholism were fed alcohol in a clinical research setting. All subjects had previously abstained from alcohol for 2–5 months. The study lasted 18 days. Patients were given 25 % of calories as protein (high), 25 % of calories as fat (low), and 50 % as carbohydrates. Alcohol was substituted for carbohydrates and was slowly increased so that after 8 days on the study, subjects were receiving 46 % of total calories as alcohol. Some subjects had interval liver biopsies during this study, and two had liver biopsies performed 1 month after alcohol was discontinued. Alcohol consumption caused histologic and biochemical fatty liver and ultrastructural changes in the liver. This occurred in spite of the fact that subjects received a nutritious diet. This highlighted the fact that alcohol itself is hepatotoxic in spite of a nutritious diet. In a second study, healthy volunteers who were never drinkers were given alcohol anywhere from 2 days to 2 weeks. Four different regimens of alcohol feeding were administered. One group received alcohol for only 2 days in addition to a standard diet, and another group received alcohol for 2 days in addition to a high-protein/low-fat diet. Importantly, both groups developed hepatic steatosis. Longer duration of feeding involved isocaloric substituting of alcohol for carbohydrates. Again, all subjects developed fatty liver on liver biopsy. These results clearly demonstrate that normal nonalcoholic subjects who regularly consume alcohol even for a relatively short period of time can develop fatty liver, and histologic changes were independent of nutritional factors.

The paramount histologic finding determining the natural history of alcoholic liver disease is alcoholic hepatitis. This is a necroinflammatory process characterized by predominant neutrophilic infiltration, ballooning degeneration of hepatocytes, and hepatocyte necrosis [113]. The development of this key clinical and histologic entity represents a "fork in the road" in relation to the natural history of ALD, with important short-term and long-term implications. Acutely, the development of alcoholic hepatitis is associated with a significant increase in proximal mortality and the potential to develop portal hypertension and its complications, even without the development of major fibrosis [114]. In the long term, for those who survive, this disease process is associated with an accelerated course of fibrosis progressing to cirrhosis in 40 % of cases [115]. These distinctions in the natural progression of alcoholic liver disease also have therapeutic implications, explaining why a subset of alcoholics with inflammatory features are candidates for treatment with anti-inflammatory agents (i.e., corticosteroids and pentoxifylline) in an attempt to reduce proximal mortality, whereas patients without these inflammatory features may be better candidates for treatment geared toward reducing long-term hepatic injury/cell death and hepatic dysfunction or possibly enhancing liver regeneration.

Due to the important acute prognostic implication of alcoholic hepatitis, multiple scoring systems have been developed to assess the severity of liver disease in terms of patient survival in order to stratify patients to proven treatment modalities. The Child–Turcotte–Pugh (CTP), the oldest scoring system, incorporates the serum bilirubin level, albumin level, PT, and the severity of ascites and hepatic encephalopathy in assigning a numerical score that is used to categorize patients (class A = scores 1–6, class B = scores 7–9, class C = scores 10–15), with a higher score denoting more severe disease [116]. This scoring

system has fallen out of favor in grading alcoholic hepatitis due to subjectivity in grading among other things. Since 2002, the United Network for Organ Sharing (UNOS) has utilized the MELD score to grade the severity of liver disease in patients awaiting liver transplant. This represents a more objective analysis, utilizing only the serum bilirubin, creatinine, and INR [117]. The use of this scoring system is a reflection of the reason for which this scoring system was initially intended to assess—the short-term prognosis of cirrhotic patients undergoing transjugular intrahepatic portosystemic shunt (TIPS) procedures [118]. The use of this scoring system has been validated in multiple studies to accurately predict the 3-month mortality of patients with liver disease, especially those patients awaiting liver transplantation [119].

The CTP and MELD scores are proven modalities to assess the gravity of liver disease due to a variety of causes. However, due to the particularly inflammatory nature and high degree of early mortality associated with acute alcoholic hepatitis, varying scoring systems have been, and continue to be, specifically developed and used in the assessment of this form of liver disease. Since its development in the late 1970s, the discriminant function (DF) of Maddrey, which incorporates the serum bilirubin and PT, is widely used to predict short-term mortality in patients with alcoholic hepatitis and to select in an evidence-based manner those who are likely to benefit from treatment with corticosteroids [120]. Patients are classified into those who have non-severe alcoholic hepatitis (DF < 32) and those who have severe alcoholic hepatitis (DF > 32). As the proximal mortality is 10 % vs. 30–60 % in the groups without treatment, respectively [121], the latter group is usually treated with corticosteroid therapy unless contraindicated [122]. A useful link to calculate 90-day mortality on acute alcoholic hepatitis, based in the MELD score, can be found at http://www.mayoclinic.org/medical-professionals/model-end-stage-liver-disease/meld-score-90-day-mortality-rate-alcoholic-hepatitis.Other specific scoring systems include the Glasgow alcoholic hepatitis score (GAHS) which is a composite of scores related to patient age, leukocyte count, serum urea levels, serum bilirubin level, and PT ratio [123], with a score greater than (9) signifying poor prognosis. In this study, patients with both, a DF > 32 and a GAHS > 9, had 28 day survival, if corticosteroid-treated vs. corticosteroid-untreated, of 78 % vs. 52 %, and an 84-day survival of 59 % vs. 38 %, respectively. If the GAHS was less than 9, there was no difference in outcome the corticosteroid treated or untreated groups. A more recent scoring system (the ABIC score) utilizing the patient's age, serum bilirubin, INR, and serum creatinine has shown promising results in the prediction of 3-month mortality in patients with alcoholic hepatitis [124]. This model stratifies the severity of alcoholic hepatitis as low (score <6.71), intermediate (score 6.71–8.99), and high (score ≥9.0). These scores correspond to a 90-day mortality of 0 %, 30 %, and 75 %, respectively.

The Lille score is unique in the fact that not only is it clinically useful in assessing the severity of patients presenting with alcoholic hepatitis, it is also used to assess the response of patients with more severe forms of alcoholic hepatitis being treated with systemic corticosteroids. The score includes 6 variables: age, albumin level, bilirubin level at day 0, bilirubin level at day 7, PT, and the presence of renal insufficiency [125]. A score of <0.45 predicts 15 % mortality and a score of ≥0.45 predicts 75 % mortality [126]. Patients with a score of ≥0.45 on day 7 while on therapy with corticosteroids are recommended to be switched over to alternative forms of treatment.

Outcomes may be highly variable once the patient has progressed to cirrhosis. Some subjects die quickly with acute and chronic liver disease; other patients who abstain from alcohol and correct other disease modifiers may live a relatively normal life, and others may do well for long periods of time only to expire from hepatocellular carcinoma. The biggest variable in this equation is continued drinking. It is well documented in many studies over the past 50 years that abstaining at any stage of liver disease (compensated or decompensated) will improve prognosis (see Fig. 8.2).

Acute-on-chronic liver failure (ACLF) is associated with a high rate of mortality. Patients develop multiple organ injury, as shown in Table 8.3. A classic characteristic of ACLF is its rapid progression, requirement for multiple organ

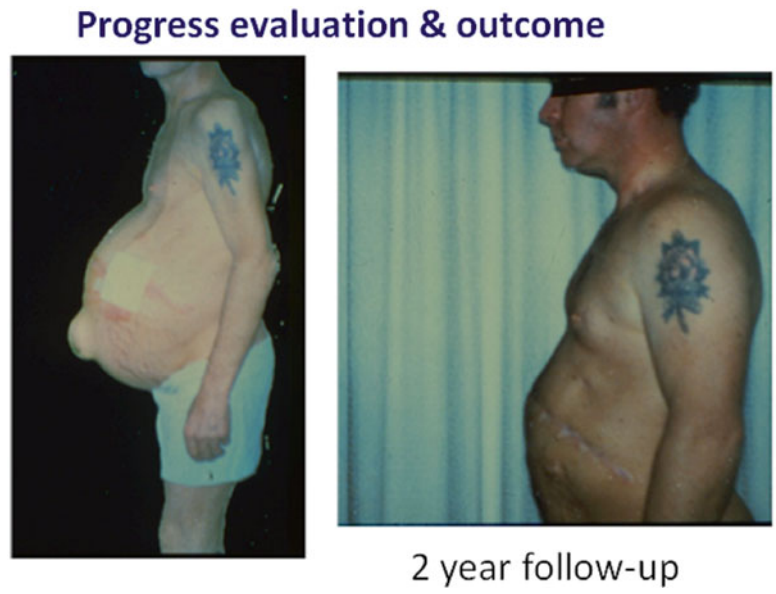

Fig. 8.2 A patient in a VA Cooperative Study with decompensated cirrhosis with superimposed AH presented with severe ascites, muscle wasting, coagulopathy, encephalopathy, etc. The second panel shows the patient after 2 years of abstinence with no ascites, a marked improvement in muscle strength, muscle mass, and functionality. This highlights the potential reversibility of even advanced ALD and the importance of abstinence

Table 8.3 Acute-on-chronic liver failure toxicity

Organ	Symptom/manifestation
Liver	Coagulopathy, ↓ synthetic function
Kidney	Acute kidney injury/hepatorenal syndrome
Intestine	Endotoxemia/dysbiosis
Lungs	Acute lung injury; pneumonia; ARDS
Heart	Cardiac dysfunction
Adrenal gland	Adrenal insufficiency
Bone marrow	Immune suppression/inflammation

support, and poor short-term and intermediate mortality of >50 %. Multiple recent reviews and scoring systems have been put forth on this topic [127–130]. A useful tool to classify and calculate in Acute-on chronic liver failure can be found at the following CLIF Consortium link: http://www.clifresearch.com/ToolsCalculators.aspx

Most studies evaluate outcome in ALD by assessing 6-month or 1-year mortalities, and data on long-term prognosis are relatively limited. A recent study from Ireland/Great Britain identified clinical and histologic factors that predict long-term (15-year) survival in outpatients with histologically advanced, non-decompensated alcoholic liver disease. The investigators studied only biopsy-proven patients with Stage 3 or Stage 4 ALD and followed them for 15 years or until death/liver transplantation. Overall, the 5-, 10-, and 15-year survival rates were 63 %, 36 %, and 24 %, respectively. In multivariate analysis, persistent drinking, smoking, age, and serum albumin at baseline were associated with increased risk of death, and persistent drinking was associated with the highest risk. Interestingly, there were no histologic features on liver biopsy that correlated with prognosis. This highlights the importance of modifying factors such as drinking and smoking cessation and other lifestyle changes that should be vigorously pursued [45].

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Nonalcoholic Fatty Liver Disease: Clinical Features, Disease Modifiers, and Natural History

9

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It has been suggested that nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome (MS), and as such, it is not surprising that NAFLD is associated with other conditions related to obesity, hyperlipidemia, and insulin resistance (IR) such as cardiovascular disease (CVD). Similarly, certain associated diagnoses have been shown to portend a worse prognosis in NAFLD populations and predict a more aggressive clinical course. This chapter will define the clinical features of NAFLD patients and the natural history of this disease as well as disease modifiers that have been associated with advanced fibrosis or a more rapidly progressive clinical course.

Clinical Presentation

NAFLD typically presents in the primary care setting as an asymptomatic elevation of liver-associated enzymes (LAEs) in a patient with features of the MS or as an incidental finding of hepatic steatosis on imaging that prompts a hepatology evaluation. NAFLD is considered to be the hepatic manifestation of the MS as defined by the presence of three or more of the following: abdominal obesity, hypertriglyceridemia, low high-density lipoprotein (HDL), hypertension, and elevated fasting plasma glucose [1].

Most studies report NAFLD to be more common in men and note a later peaking prevalence in women [2]. NAFLD is found in all ethnic groups, although there is some evidence to suggest it is more common among certain ethnic groups. The Dallas Heart Study suggested that Hispanics had the highest prevalence of NAFLD at 45 % compared to 33 % of Caucasians and 24 % of African Americans [3]. Similar findings were reported by Williams et al. with a 58.3 % Hispanic NAFLD prevalence compared to 44 % for Caucasians and 35 % for African Americans, and the majority of studies to date have confirmed Hispanics have higher rates of NAFLD, particularly in comparison to African Americans. A recent study suggested Hispanic ethnicity was an independent risk factor of NASH (OR 1.72, CI 1.28–1.33) in contrast to the inverse association seen with NASH and African Americans (OR 0.52, CI 0.34–0.78) [4]. Asian populations appear to have a similar prevalence to their Caucasian counterparts with one study suggesting a prevalence of 27 % of urban Chinese [5]. This apparent association with ethnicity requires further clarification. Research focusing on specific genes

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Table 9.1 Clinical and laboratory features of nonalcoholic fatty liver disease

Symptoms	Signs	Laboratory features
Common		
– None	– Hepatomegaly	– Two- to fourfold ↑ of serum ALT and AST – AST/ALT ratio <1 usually – Alkaline phosphatase level ↑ – Normal bilirubin, albumin levels, and prothrombin time – ↑ Serum ferritin level
Uncommon		
– Vague RUQ pain – Fatigue – Malaise	– Splenomegaly – Spider angiomata – Palmar erythema – Ascites	– Low-titer (less than 1:320) ANA

ANA antinuclear antibodies, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *RUQ* right upper quadrant

predictive of NAFLD and/or NASH is ongoing which may clarify this association. In the interim, it is important to consider NAFLD in all ethnicities while keeping a higher index of suspicion when evaluating those of Hispanic descent.

The majority of patients with NAFLD are asymptomatic, but some may describe vague right upper quadrant pain, fatigue, and malaise (Table 9.1). The presence of symptoms does not appear to be associated with disease severity, and symptoms are not a reliable indicator of the presence or absence of significant necroinflammation. While hepatomegaly is common, it may be difficult to appreciate on physical examination because of obesity. Obesity can be categorized based on the location of fat depositions with the specific finding of dorsocervical lipohypertrophy (DCL) thought to be a novel finding in NAFLD that may correlate with disease severity [6]. Patients with NASH cirrhosis can demonstrate the typical stigmata of chronic liver disease such as splenomegaly, spider angiomata, and ascites, but these findings are not seen in the absence of cirrhosis.

As clinical, laboratory, and liver biopsy findings are similar in alcoholic liver disease, the diagnosis of NAFLD can only be made in the absence of significant alcohol use. This is typically defined as the consumption of less than 20–40 g of alcohol per day. Other conditions that lead to hepatic steatosis independent of NAFLD are seen less commonly than alcoholic liver disease, but still should be considered. Hepatic steatosis can occur in conditions resulting in rapid weight loss as seen with total parenteral nutrition, extensive small bowel resection, biliopancreatic diversion, or jejunoileal bypass. Medications such as amiodarone, valproic acid, methotrexate, tamoxifen, glucocorticoids, certain antiretrovirals, and tetracyclines as well as systemic conditions such as Wilson disease, abetalipoproteinemia, and lipodystrophy can also produce hepatic steatosis.

A mild-to-moderate (1.5–4-fold) elevation of the serum aspartate aminotransferase (AST) or alanine aminotransferase (ALT) level, or both, is common, although levels exceeding ten times the upper limit of normal are rare. A mean ALT of 83 and AST of 63 IU/mL was shown in a large retrospective study of NAFLD patients [7]. It is important to recognize that NAFLD can occur with completely "normal" LAEs and laboratory findings do not always correlate with the histologic severity of NAFLD. The entire histologic spectrum of NAFLD, including cirrhosis, can be seen in patients with normal or near-normal serum aminotransferase levels [8]. In most NAFLD patients, the serum ALT level usually is greater than the AST level, particularly in comparison to alcoholic liver disease where AST is typically twofold higher than ALT. The alkaline phosphatase and gamma-glutamyl transpeptidase (GGT) levels may also be elevated, and occasionally, NAFLD can present as an isolated alkaline phosphatase elevation, more often in female patients [9]. The serum bilirubin level, prothrombin time, and serum albumin level are

usually normal, except in patients with NAFLD-associated cirrhosis.

Antinuclear antibodies (ANA) can be elevated in up to one fourth of patients with NAFLD, although typically in low titers less than 1:320. Laboratory tests for other chronic liver disease are negative [10], although NAFLD can coexist in patients with hepatitis C infection (HCV). In HCV and in one study of 628 adult NAFLD patients, serum ferritin > 1.5 × upper limit of normal was independently associated with a higher NAFLD activity score (NAS) [11]. Iron overload in NAFLD populations appears to be secondary and limited to Kupffer cells, with a subsequent study demonstrating that the frequency of genetic hemochromatosis was not increased in NAFLD patients [12]. This is consistent with the belief that increased serum iron indices are a by-product of hepatic inflammation, rather than a direct contributor to the pathogenesis of NAFLD. A recent study supported this argument and demonstrated that 6 months of phlebotomy with the goal of lowering serum ferritin did not change serum markers of inflammation or LAEs in a study of 74 NAFLD patients [13]. Alternatively, two older small studies have suggested that iron depletion may have a therapeutic role in NAFLD by decreasing plasma insulin, glucose, and serum aminotransferase levels [14, 15]. Further study is likely necessary to clarify if there is any therapeutic benefit to phlebotomy, but there is consensus that elevated ferritin is associated with increased disease activity (although not necessarily fibrosis) in NAFLD populations.

In the absence of a liver biopsy, it is difficult to distinguish those patients with NASH as the most clinically relevant subset of patients with NAFLD in terms of liver-specific outcomes. As previously mentioned, the clinical picture and symptom profile are decidedly unhelpful in elucidating which patients meet criteria for NASH. Noninvasive tests to include laboratory and radiographic modalities as well as scoring systems will be discussed elsewhere, although they generally have proven most useful in identifying advanced fibrosis. A clinical presentation of NAFLD that includes borderline low platelets or an AST:ALT ratio approaching 1 increases the clinical likelihood of advanced fibrosis and should warrant further work-up either with noninvasive testing or a liver biopsy.

Clinical Associations

NAFLD is associated with a wide variety of clinical conditions, and often their coexistence has clinical implications (Fig. 9.1). The most studied and well-founded association is that of NAFLD with diabetes mellitus (DM). NAFLD and specifically nonalcoholic steatohepatitis (NASH) are often associated with DM where a 60–76 % prevalence of NAFLD and a 22 % prevalence of NASH have been reported [16]. NAFLD appears to increase the risk of developing DM [17], and DM is an independent risk factor for NAFLD [18]. The presence of both conditions has significant clinical implications as it is predictive of increased risk of death from both liver-related

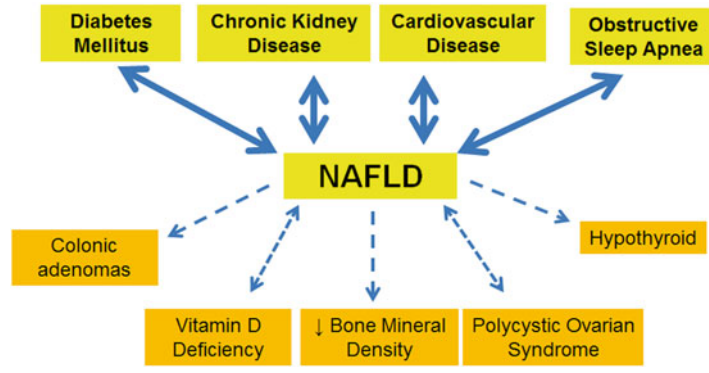

Fig. 9.1 Conditions associated with NAFLD. This figure illustrates conditions associated with NAFLD. A *bidirectional arrow* indicates a mutual association. A *dashed arrow* indicates mixed data but at least some association with *direction of arrow* indicating prevailing relationship

and all-cause mortalities [19]. NAFLD also has been associated with higher rates of end-stage organ damage from DM as evidenced by proliferative retinopathy and increased rates of chronic kidney disease (CKD) [20].

CVD comes in a strong second with numerous studies showing increased rates of CVD and cardiovascular events in NAFLD patients. Carotid artery intima-media thickness has been shown to be increased in NAFLD patients [21], and a meta-analysis of eight studies confirmed NAFLD as an independent risk factor for CVD with an OR of 2.05 (95 % CI, 1.81–2.31) [22]. CVD is the primary cause of death in NAFLD patients, but the mechanisms defining this relationship are still under investigation. Epicardial fat has been recently shown to be increased in NAFLD patients and proportionally related to hepatic fibrosis so this may partially explain the association [23]. Other potential explanations for the link between NAFLD and CVD include inflammation, oxidative stress, insulin resistance, dyslipidemia, endothelial dysfunction, and cytokine imbalances [24]. Interestingly, the risk for CV events persists even after liver transplant with the largest study to date demonstrating a 4.12 OR (95 % CI, 1.91–8.90) for CV events in NAFLD patients compared to patients transplanted for alcoholic liver disease [25].

Obstructive sleep apnea (OSA) has also been shown to be prevalent in NAFLD populations at rates higher than the estimated general OSA prevalence of 1–4 % or even the 25–35 % shown in obese populations [26, 27]. Fifty percent of NAFLD patients have symptoms suggestive of OSA, and 90 % of obese patients with OSA have NAFLD [28]. There is even some evidence to suggest an association of the chronic intermittent hypoxia seen in OSA with NAFLD disease severity. LAE elevation in a cohort of patients with steatosis on ultrasound was shown to be related to the oxygen desaturation index that is used to determine severity of OSA [29]. A study of morbidly obese patients undergoing bariatric surgery demonstrated OSA was associated with a high NAS and increased fibrosis [30]. This was also shown in a study of pediatric patients which demonstrated increased fibrosis in NAFLD patients with OSA [31]. It is less clear whether treatment of OSA with continuous positive airway pressure (CPAP) or other modality would benefit NAFLD. One study of obese males did demonstrate an improvement in LAEs with CPAP [32], although this was not confirmed by a similar subsequent study [33]. Histologic outcomes were absent from both of these studies, and larger randomized controlled trials with histologic end points are required to definitely evaluate the effectiveness of OSA treatment in NAFLD.

CKD has long been associated with hypertension and DM and is common in the general population with reported prevalence rates ranging from 4.3 to 13 % [34, 35]. Higher prevalence rates on the order of 21–54 % have been demonstrated in NAFLD populations where CKD was defined by a decrease glomerular filtration rate (GFR) $\leq$60 mL/min/1.73 m^2, overt proteinuria, or microalbuminuria with a urinary albumin/creatinine $\geq$30 mg/g [36]. In seven studies, NAFLD was independently associated with CKD after adjusting for age, sex, body mass index, hypertension, DM, smoking, and hyperlipidemia. One important caveat was that most of these did not use liver biopsy to evaluate for NAFLD and instead relied on LAEs or ultrasound (US). Despite the preponderance of evidence linking CKD and NAFLD, one large cross-sectional study using NHANES data from 1988 to 1994 did not show an association of CKD with a US diagnosis of NAFLD after adjusting for components of the metabolic syndrome [37]. A subsequent NHANES analysis from 2001 to 2006 showed mild elevation of GGT was associated with an increased prevalence of CKD [38] and was further supported by three hospital-based studies using liver biopsy to evaluate for NAFLD [39–41]. These smaller studies were strengthened by their histologic data and generally showed NAFLD, and in some instances NASH or advanced fibrosis, to be associated with CKD. The association of NAFLD and CKD was confirmed by the large meta-analysis conducted by Musso et al. that contained 33 studies with over 63,000 participants, and the severity of each diagnosis was increased in the presence of the associated diagnosis [42].

The evidence demonstrating that a diagnosis of NAFLD carries an increased risk of incident

CKD is equally compelling. Four of the five studies to date demonstrated that NAFLD was independently associated with the development of de novo CKD, although it is notable that three of the five studies used elevated GGT to diagnose NAFLD [43–47]. The four positive studies demonstrated HRs ranging from 1.49 to 4.38 for the risk of developing CKD in the presence of NAFLD.

The relationship of CKD and NAFLD in the setting of cirrhosis necessitating liver transplant requires special mention as the number of transplants for NASH cirrhosis increases. A myriad of issues this presents are addressed in a recent editorial by Musso et al. which summarized the rising rates of liver transplant for NASH cirrhosis along with increasing postoperative issues with CKD and renal failure [48]. The need for dual organ transplantation (liver and kidney) was also notable in this population, and chronic kidney disease has been shown to be associated with increased mortality in liver transplant [49].

In summary, NAFLD and CKD are strongly associated with abundant evidence linking these two diagnoses and their respective disease severities. Patients diagnosed with NAFLD should be evaluated for CKD, and conversely those with CKD, evaluated for NAFLD.

The relationship between vitamin D and NAFLD has also been the focus of extensive investigation with most evidence suggesting vitamin D deficiency (VDD) was found more commonly in NAFLD populations [50]. VDD has shown increased and widespread prevalence in recent years similar to that of NAFLD prevalence rates, although the coexistence of NAFLD and VDD appears to go beyond a simple association. VDD is found in populations with NAFLD at higher rates than matched controls when using data from NHANES II [51]. Further study has shown this association to be independent of age, gender, and triglyceride or glucose levels [52]. The association of VDD and disease severity is more controversial, although one study showed lower VDD levels in NASH patients compared to isolated fatty liver [53]. This was not substantiated by a subsequent study [54] and definitive evidence is still required. There are no prospective studies to suggest that vitamin D replacement may improve NAFLD or NASH, although it is reasonable to check vitamin D levels and replete as necessary in known NAFLD patients.

Bone mineral density (BMD) in the setting of NAFLD has also been investigated with most studies suggesting an inverse relationship. A study of postmenopausal Korean women demonstrated lower BMD in US-defined NAFLD even with adjustment for BMI, smoking, age, alcohol use, and the metabolic syndrome [55]. This was also shown in a male Chinese population where those with US-diagnosed NAFLD were 2.5 times more likely to have an osteoporotic fracture [56] and in a smaller study of children, where 45 % with biopsy-proven NAFLD had low BMD compared to 0 % of age- and weight-matched controls [57]. No data exists as to whether or not osteoporosis is associated with advanced histology in NAFLD. Similar to VDD, a diagnosis of NAFLD should increase suspicion for coexistent osteoporosis, although it is too early to advocate universal BMD testing in this large population.

Components of the MS including obesity, insulin resistance, and dyslipidemia have also been shown to be associated with increased prevalence of colonic adenomas. It is therefore not surprising that several retrospective studies and one prospective study have demonstrated a relationship between NAFLD and colonic adenomas. The two largest studies to date, both in Asian populations, showed a higher prevalence of colonic adenomas as well as advanced neoplasia such as cancer, high-grade dysplasia, or villous histology in NAFLD populations [58, 59]. In these two studies, NASH histology was more strongly correlated to adenoma detection than non-NASH NAFLD. The largest American study to date confirmed an association of adenomatous polyps in NAFLD compared to non-NAFLD populations, but this did not correlate to histology [60]. The association of adenomatous polyps and NAFLD did not appear to extend to colorectal cancer (CRC) where 227 patients with CRC were followed (27 % with NAFLD) and outcomes were similar among NAFLD and non-NAFLD groups [61].

Polycystic ovarian syndrome (PCOS) is another condition that has been associated with NAFLD.

Markedly increased rates of NAFLD in PCOS patients with OSA (83.3 % vs. 26.9 %, $p<0.01$) have been demonstrated [62] as well as in upward of 15 % of obese adolescent females [63]. Subsequent study associated the increased risk of PCOS patients for NAFLD in a manner independent of BMI [64]. A cross-sectional Australian study has provided evidence that the converse is also true: NAFLD patients are at increased risk of PCOS. In this small study, ten of 14 patients with US- or biopsy-proven NAFLD had PCOS which translated to a 71 % prevalence, significantly higher than a similar female population [65]. At a minimum, NAFLD should be considered in all PCOS patients, particularly those with elevated LAEs, and female NAFLD patients with gynecologic symptoms should be evaluated for PCOS.

Other endocrine-related disorders associated with NAFLD include hypothyroidism, growth hormone deficiency, hypogonadism, hypopituitarism, and hypercortisolemia. The data is most abundant linking NAFLD with higher prevalence of hypothyroidism. Biopsy-proven NAFLD has been associated with a 21 % prevalence of hypothyroidism compared to 9.5 % of age-, sex-, ethnicity-, and BMI-matched controls [66]. A larger study also confirmed this association in patients with both overt and subclinical hypothyroidism in a manner independent of known metabolic risk factors [67].

Another association with NAFLD that has been seen at least in preliminary studies is elevated uric acid levels. The association of elevated uric acid and NAFLD was demonstrated in a cohort of 528 Chinese postmenopausal women of normal BMI [68] which was confirmed in group of biopsy-proven male NAFLD patients [69]. Further study is required to determine if elevated uric acid levels translate to clinically significant gout and whether hyperuricemia is associated with increased disease severity in NAFLD.

In total, NAFLD is associated with a myriad of extrahepatic conditions, most of which are also related to the MS. The association of NAFLD with intra- and extrahepatic malignancy is discussed elsewhere in this text but is also thought to be related to inherent risk from some component of the MS or obesity.

Natural History

The natural history of NAFLD is highly variable, particularly since disease progression does not always follow a linear course. Histopathology remains critically important, and even though the accepted paradigm of non-NASH NAFLD versus NASH is overly simplistic, this distinction is still an important predictor of natural history and outcomes. Obtaining a liver biopsy allows for the identification of features such as lobular and portal inflammation and hepatocyte ballooning that enables a pathologist to distinguish non-NASH NAFLD from NASH, and in addition, it allows for the quantification of fibrosis. The prognosis in patients with steatosis, none to mild nonspecific hepatocellular inflammation, and no fibrosis (non-NASH NAFLD) has been thought to be favorable with minimal potential for histologic or clinical progression [70, 71]. Most non-NASH NAFLD patients are thought to have similar mortality rates to the general population, while an established diagnosis of NASH predicts a reduced life expectancy from cardiovascular, malignancy, or liver-related causes [72, 73]. Fortunately, it is estimated that approximately 70–75 of adult NAFLD patients will fit into the non-NASH NAFLD category [74] (Fig. 9.2).

Recent evidence from a natural history study following 108 NAFLD patients for a median of 6.6 years revealed 44 % of non-NASH NAFLD patients progressed to NASH and 22 % alarmingly progressed to stage 3 fibrosis [75]. Overall, 37 % of non-NASH NAFLD and 43 % of NASH patients from this cohort had some degree of fibrosis progression during follow-up. While this relatively small study does not provide definitive evidence, it certainly calls into question the previous dogma that non-NASH NAFLD does not progress to NASH or lead to significant fibrosis. Further evidence that non-NASH NAFLD can lead to hepatic fibrosis was also revealed in a meta-analysis of 11 cohort studies that included 411 patients with biopsy-proven NAFLD. Non-NASH NAFLD patients with stage 0 fibrosis at baseline progressed 0.07 stages versus 0.14 stages annually in NASH patients [76]. The authors translated this into one stage of fibrosis

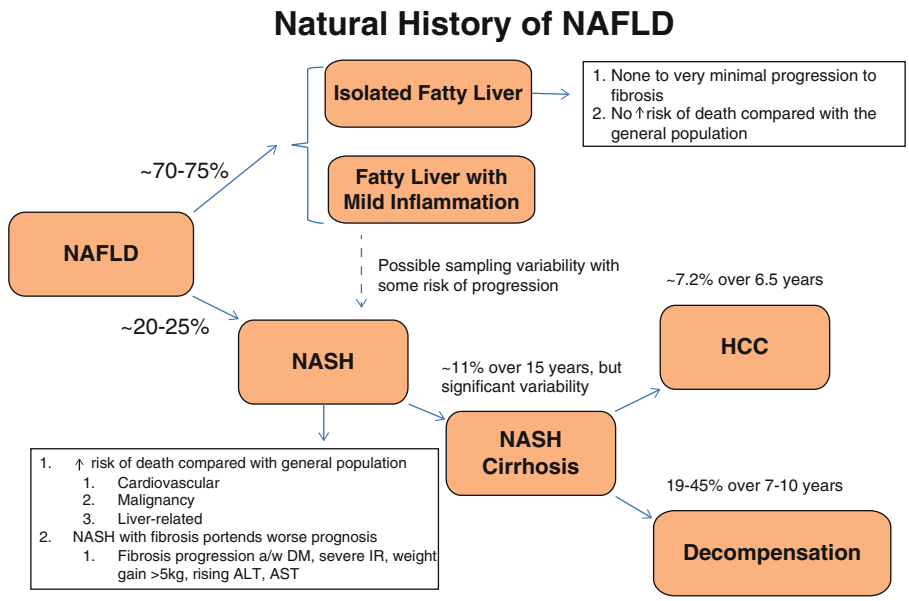

Fig. 9.2 Natural history of NAFLD. The progression of cirrhosis to end-stage liver disease is 39–62 % in patients with NASH. Of these patients, 22–33 % will experience liver disease-related mortality. The survival time of these patients is 5–7 years. The development of ascites is the most common liver-related morbidity. The mortality rate is higher than in the general population (standard mortality ratio, 1.34; 95 % CI: 1.003–1.76; $p=0.03$) (Adams LA et al. *Gastroenterol.* 2005; 129:113–121 abst). The study by Sanyal et al. compared outcomes in patients with cirrhosis in NASH vs. cirrhosis in HCV, using the Child-Turcotte-Pugh (CTP) score. The CTP score assesses the severity of liver disease by scoring bilirubin, ascites, prothrombin time (INR), encephalopathy grade, and serum albumin. NASH patients with CTP class A had a lower mortality rate than HCV patients. Mortality rates were similar in NASH and HCV at CTP class B and higher. These results corroborate the Hui et al. study which found that NASH-associated cirrhosis had a similar prognosis to HCV cirrhosis. Modified from Torres DM et al. [2], with permission of Elsevier

progression over 14.3 years for a non-NASH NAFLD patient and 7.1 years for a patient with NASH. Another key finding was the significant variability in fibrosis progression rates with approximately one in five patients demonstrating rapid fibrosis progression, although due to limited data, the unifying factors associated with rapid progression could not be identified. These findings are somewhat consistent with the previous teaching that one third of NASH patients improve, one third stay the same, and one third worsen with the additional caveat that non-NASH NAFLD patients may also be susceptible to disease progression.

There is growing evidence that the non-NASH NAFLD paradigm is too broad in scope as it relates to prognosis and natural history. Data suggest that non-NASH NAFLD can possibly be further divided into an isolated steatosis group, having no inflammation, and a group defined by fatty liver and mild inflammation, or "indeterminate NASH" as proposed in a recent editorial [77]. Despite the mild inflammation, ballooning is not apparent and thus a diagnosis of NASH cannot be made. This further delineation of NAFLD patients is supported by a recent study showing that isolated steatosis patients progress much more slowly than patients with steatosis and mild inflammation [78] and two prior studies that demonstrated that isolated steatosis patients did not progress at all [72, 79].

With preeminence of NASH versus non-NASH NAFLD now in question based on recent data, the importance of fibrosis in predicting outcomes has remained. NASH with fibrosis suggests a worse prognosis than NASH without fibrosis [80]. Some studies have suggested fibrosis is the most important predictor of outcome

exceeding the NAS which includes necroinflammation, ballooning, and degree of steatosis. A large natural history study of 229 biopsy-proven NAFLD patients followed for a mean of 26.4 years (±5.6, range 6–33) demonstrated that NAFLD with fibrosis portended a worse prognosis, while the NAS was not helpful in natural history determination [81]. Increased fibrosis stages 3–4, regardless of the NAS, had substantially increased mortality with a HR 3.3 (CI 2.27–4.76, $p<0.001$). Conversely, a high NAS in the absence of advanced fibrosis did not predict outcomes. While this study did not distinguish NASH from non-NASH NAFLD, the authors grouped patients into NAS 0–4 or 5–8 as a (suboptimal) surrogate. A NAS of 0–4 or 5–8 in the absence of advanced fibrosis (stage 0–2) did not predict increased overall mortality with an HR of 1.41 (CI 0.97–2.06, $p=0.07$) and HR 1.13, respectively (95 % CI 0.79–1.60, $p=0.51$). The caveat to this was two patients with a NAS 0–4 died due to cirrhosis-related complications, although both patients had stage 2 fibrosis. The authors explained these findings in the discussion suggesting that the NAS was overly reliant on hepatic steatosis to which it gives equal importance alongside ballooning and lobular inflammation.

Additional factors associated with fibrosis progression include the presence of DM, severe insulin resistance, cigarette smoking, weight gain greater than 5 kg, or rising ALT and AST levels [82, 83]. As previously mentioned, fibrosis progression rates are variable, and no clinical or laboratory data has been shown to reliably predict disease course. One author suggested that ~11 % of NASH patients progress to cirrhosis over a 15-year period [84]. Interestingly, despite the similarities in NAFLD histology to alcoholic hepatitis, outcomes in NAFLD are much better. The 5-year survival rate of patients with alcoholic hepatitis is only 50–75 %, in large part due to the development of cirrhosis in greater than 50 % with its inherent complications [85].

Cirrhosis secondary to NAFLD has comparable outcomes to other causes of cirrhosis, and most cryptogenic cirrhosis is thought to be NAFLD [86]. NAFLD cirrhosis can lead to hepatocellular carcinoma (HCC) and NAFLD-related HCC is the fast-growing indication for liver transplantation [87, 88]. Five- to ten-year outcomes of NAFLD-associated cirrhosis appear similar to that for HCV-associated cirrhosis [89].

The third most common indication for orthotopic liver transplant (OLT) behind chronic hepatitis C and alcoholic liver disease in America is NASH cirrhosis, although it is expected to become the number one indication for liver transplant with the next 1–2 decades [90]. Long-term survival from a transplant due to NASH cirrhosis is similar to other indications although eligibility for transplant may be limited secondary to coexisting conditions such as heart disease and 30-day transplant mortality is still higher for NASH cirrhosis [91, 92]. The majority of patients have recurrent steatosis 5 years out from transplant, although only 5 % developed recurrent cirrhosis within that time [93]. Recent data distinguished two kinds of posttransplant NAFLD—that of de novo fatty liver disease with an alternative indication for the primary liver transplant and recurrent NAFLD [94]. Recurrent NAFLD in this study had a more aggressive course with 71 % of patients showing stage 3–4 fibrosis after 5 years compared to 12.5 % ($p<0.02$) of those with de novo NAFLD. This combined with the previously mentioned almost fourfold increased risk for cardiovascular events posttransplant (compared to transplant for alcoholic liver disease) necessitates the need to develop effective screening methods for NASH cirrhotics to identify those at risk for recurrent NASH or CVD posttransplant.

Conclusion

NAFLD can have a varied clinical presentation with most patients demonstrating an asymptomatic elevation of serum aminotransferases with hepatic steatosis on imaging. Occasionally, symptoms may be what bring that patient to medical attention although it is important to recognize that symptoms do correlate to disease severity or disease progression. Clinical suspicion for NAFLD and in particularly NASH should be heightened in a number of medical conditions associated with the MS. First and

foremost, diabetic patients, particularly those with elevated serum aminotransferases, should be evaluated for NAFLD. Obesity, CVD, and OSA are also associated with NAFLD and are thought to increase the likelihood of NASH.

The natural history of NAFLD is diverse with some patient showing a wholly benign clinical course while others rapidly progressing to cirrhosis. Liver biopsy is our best tool to determine who is at the greatest risk of disease progression although it only provides data for one point in time in a disease that can either improve, stay the same, or worsen. Most experts agree that absent fibrosis is a good albeit not perfect prognostic sign and that non-NASH NAFLD usually predicts a better outcome than NASH histology. The more recent findings that have suggested that NAFLD without NASH can still become NASH have shifted the natural history paradigm (see Fig. 9.2), and we are left to fall back on fibrosis data as a measurable data point that correlates well with outcomes, particularly in the setting of no (F0) or abundant (F4) fibrosis.

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10 Diagnostic Approaches and Clinical End Points of Treatment in Alcoholic Liver Disease

Jaeyoun Cheong, Eva Stein, and Ramon Bataller

Abbreviations

ABIC	Age, bilirubin, international normalized ratio, and creatinine
AH	Alcoholic hepatitis
AHHS	Alcoholic hepatitis histological score
ALD	Alcoholic liver disease
ASH	Alcoholic steatohepatitis
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
AUDIT	Alcohol Use Disorder Inventory Test
CAP	*Controlled attenuation parameter*
CDT	Carbohydrate-deficient transferrin
CT	Computed tomography
DF	Discriminant function
GGT	γ-Glutamyl-transpeptidase
HCC	Hepatocellular carcinoma
HSC	Hepatic stellate cells
INR	International normalized ratio
MELD	Model for End-Stage Liver Disease
MARS	Molecular adsorbent recirculating system
MRI	Magnetic resonance imaging
NASH	Nonalcoholic steatohepatitis

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Introduction

Alcoholic liver disease (ALD) is one of the main causes of chronic liver disease worldwide and can lead to fibrosis and cirrhosis [1]. Alcohol-related liver deaths account for up to 48 % of cirrhosis-associated deaths in the United States [2] and are also major contributors to liver disease-related mortality in other countries [3]. The spectrum of ALD includes simple steatosis, alcoholic steatohepatitis (ASH), fibrosis, cirrhosis, and superimposed hepatocellular carcinoma (HCC). In addition, patients with underlying ALD can develop episodes of jaundice and clinical decompensation called alcoholic hepatitis (AH). The molecular and cellular mechanisms of ALD have been partially identified. Early studies suggested that ethanol metabolism-associated oxidative stress and malnutrition and the activation of the adaptive immune response play a major role in ALD. More recent studies implicate ethanol-mediated induction of gut-derived endotoxin and subsequent activation of innate immune response in the pathogenesis of ALD [4–9].

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Compared to the recent advances in viral hepatitis, few advances have been made in the management of patients with ALD [10]. To date, the only effective therapy to attenuate the clinical course of ALD and even reverse liver damage is prolonged alcohol abstinence. No new drugs for ALD have been successfully developed since the early 1970s, at which time the use of steroids was proposed for the treatment of severe AH [11]. The lack of advances in the field of ALD is due to intrinsic difficulties in performing clinical trials in patients with active addiction, a poor knowledge of molecular drivers in humans, and the lack of experimental models of advanced ALD [12, 13]. Therefore, there is an urgent need to develop new pathophysiology-oriented therapies. In addition, a delineation of its natural history and prognostic factors, as well as the development of reliable noninvasive markers, is required.

The clinical end points of therapy in patients with ALD depend on the baseline condition. In patients with early asymptomatic phenotypes (fatty liver and ASH with or without mild fibrosis), the end points consist of normalization of laboratory abnormalities and resolution of fibrosis. There is a clear need to develop noninvasive tools to monitor the response to therapy in patients with early ALD. In contrast, patients with severe forms such as decompensated cirrhosis and/or AH often have severe complications leading to early mortality. Therefore, the main clinical end point in these patients is short-term survival. Other important end points are the reduction in the occurrence and severity of clinical complications and the improvement of tests indicative of liver failure (i.e., MELD score). In this chapter, we describe the phenotypes, natural history, and diagnostic approaches as well as management of patients with ALD.

Clinical Phenotypes in ALD

The clinical types of ALD are quite heterogeneous, ranging from early asymptomatic forms to life-threatening conditions such as AH. ALD includes a broad spectrum of disorders from fatty liver to more severe forms of liver injury, including ASH with or without progressive fibrosis, cirrhosis, AH, and superimposed HCC (Fig. 10.1).

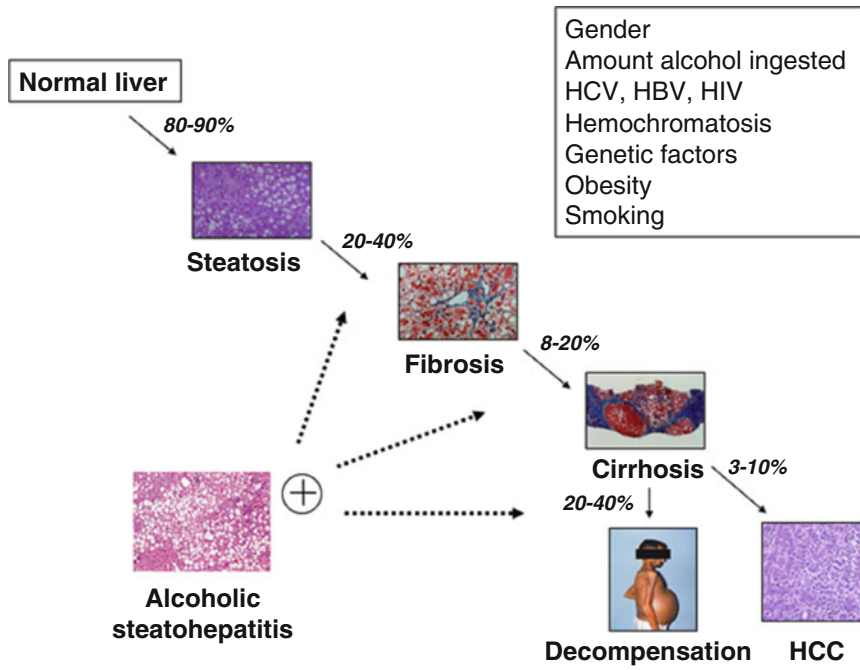

Fig. 10.1 Spectrum of alcoholic liver disease and main modifying factors. The percentage represents the patients who progress from one stage to the next

Fatty liver, which is the earliest consequence of alcohol abuse, develops in up to 90 % of heavy drinkers [14]. It is usually asymptomatic and is considered to be fully reversible after prolonged abstinence. In 30–35 % of patients with continuous hazardous alcohol consumption, fatty liver progresses to more severe forms of ALD such as ASH [15]. Eventually, patients develop progressive liver fibrosis (20–40 %) and cirrhosis (8–20 %), which confers a high risk of complications related to liver failure and portal hypertension, such as ascites, variceal bleeding, and hepatic encephalopathy [16, 17].

Alcohol-induced steatosis is characterized by the accumulation of fat (mainly triglycerides, phospholipids, and cholesterol esters) in hepatocytes. The natural history of simple steatosis is not well known, but is considered to be a benign condition. Whether it predisposes to extrahepatic consequences such as diabetes or cardiovascular disease is unknown. It has been estimated that a third of patients with steatosis will develop hepatic inflammation (ASH) if they are persistent in drinking abusively [18]. ASH is a syndrome characterized by inflammatory cell infiltration of the liver (mostly PMN) and hepatocellular injury. ASH includes a spectrum of diseases ranging from mild damage to severe, life-threatening injury [17, 19]. Patients with ASH can develop progressive fibrosis. Liver fibrosis represents a wound-healing response to repeated liver injury. The persistence of ASH over a long period may accelerate the progression of fibrosis, yet the precise natural history of this condition is not well known [20]. The most advanced stage of fibrosis is micronodular cirrhosis, which may be mixed with macronodular in some patients [21]. The clinical course of alcoholic cirrhosis is similar to other types of liver disease. Alcoholic cirrhosis can rapidly progress to end-stage liver disease if the patients continue drinking. Due to immune paralysis, ALD cirrhotics are predisposed to severe infections that carry bad prognoses [22].

Patients with advanced ALD and active drinking can develop an episode of superimposed AH [13]. AH is a life-threatening condition characterized by an abrupt increase in bilirubin and other parameters of liver synthetic dysfunction as well as severe portal hypertension. In severe cases and in patients with liver cirrhosis, AH leads to severe complications related to liver failure and portal hypertension and carries a high short-term mortality [17].

The modifying factors for ALD are not well known and are based on few retrospective studies that identified several risk factors for the susceptibility of ALD. These include gender, obesity, drinking patterns, dietary factors, non-sex-linked genetic factors, and cigarette smoking [19, 23, 24]. Female sex is a well-documented risk factor for susceptibility to ALD, likely due to lower levels of gastric alcohol dehydrogenase, a higher proportion of body fat, and the presence of estrogens. The amount and type of alcohol consumed, drinking patterns, dietary factors, and cigarette smoking have been shown to influence the risk of developing ALD cirrhosis [25]. Current attention is being paid to the deleterious effects of "binge drinking," which is particularly common in [26] the young population [27]. Obesity is ,another factor that can synergistically accelerate fibrosis progression and cirrhosis development in heavy drinkers [28, 29]. The mechanisms of such interactions are not well described. In the experimental setting, a high-fat diet exacerbated several of the pathogenic consequences of alcohol, including ER stress and macrophage activation [30]. The genetic factors that influence an individual's susceptibility to the development of advanced ALD are largely unknown. Variations in genes encoding antioxidant enzymes rather than cytokines and alcohol-metabolizing enzymes seem to play a role [24]. As occurs in NAFLD, adiponutrin (PNPLA3) is a major modifier of ALD [26]. Finally, chronic drinking synergistically accelerates progression of liver diseases in the presence of comorbid factors such as hepatitis virus B or C and/or HIV infection, nonalcoholic fatty liver disease, hemochromatosis, etc. For example, it is well documented that alcohol consumption and viral hepatitis often coexist and synergistically accelerate the progression of liver fibrosis, cirrhosis, and HCC [31, 32].

Diagnostic Approaches in ALD

Clinical, Analytical, and Imaging Diagnosis of ALD

Diagnosis of early-stage ALD is based on the combination of clinical, laboratory, and imaging findings. With adequate history of excessive drinking and analytical evidence for liver disease, the diagnosis of ALD can be established without the need of histological confirmation in most cases. Unfortunately, symptoms often develop only after severe, life-threatening liver disease has already developed. There is an urgent need to develop programs for the early detection of ALD in primary care centers and alcohol addiction clinics. A careful medical history from the patient and close relatives is needed to obtain information on the amount, frequency, duration, and type of drinking. Obtaining an accurate alcohol use history in patients with suspected ALD can be difficult, since many patients underreport their alcohol consumption. In some cases, speaking with the patient's family or close friends may help in obtaining a more precise history. Underreporting should be suspected if stigmata of alcoholism, compatible laboratory findings, or other affected organs are present. A structured questionnaire should be administered to obtain more qualitative information about a patient's alcohol consumption and problems. Among the different existing questionnaires, the AUDIT (Alcohol Use Disorder Inventory Test) remains the "gold standard" tool [33].

At physical exam, patients with steatosis may have a normal examination or hepatomegaly. Patients with AH typically present with jaundice, and patients who have developed cirrhosis may have peripheral stigmata of liver disease, splenomegaly, or signs of hepatic decompensation. Physical examinations may also show evidence of chronic alcohol consumption (vascular ectasia, parotid hypertrophy, Dupuytren's contracture, sarcopenia, signs of peripheral neuropathy, rhinophyma, etc.) (Table 10.1). These signs can be useful to identify ALD in patients that underreport the abusive alcohol consumption.

Table 10.1 Physical examination findings in patients with alcohol use disorder

Abdominal wall collaterals
Ascites
Cutaneous telangiectasias
Digital clubbing
Disheveled appearance
Dupuytren's contractures
Gynecomastia
Hepatomegaly
Jaundice
Malnutrition
Palmar erythema
Parotid enlargement
Peripheral neuropathy
Rhinophyma
Spider angiomata
Splenomegaly
Testicular atrophy

For patients with a history of alcohol misuse and evidence of liver disease, further laboratory tests should be done to exclude other etiologies and to confirm diagnosis. Laboratory tests that should be obtained in patients with suspected ALD include liver function tests, a complete blood count, serum albumin, and coagulation studies (prothrombin time, INR). There are no laboratory tests that reliably differentiate ALD from other causes of liver disease. The most common pattern of liver biochemical test abnormality in ALD is a disproportionate elevation of the aspartate aminotransferase (AST) compared with the alanine aminotransferase (ALT), resulting in a ratio of AST to ALT greater than 1 [34, 35]. AST to ALT ratio >2 is highly suggestive of ALD, although it can also be found in advanced cirrhosis of any etiology [36]. A typical finding in patients with ALD is elevated γ-glutamyltranspeptidase (GGT). However, GGT activity is not specific for ALD and can be also elevated by other conditions such as cholestatic liver disease, cardiac insufficiency, and drugs such as antifungals or anticonvulsants. Another biomarker indicative of alcohol abuse is carbohydrate-deficient transferrin (CDT) [37]. A combined index including GGT and CDT can be useful in detecting hazardous alcohol intake [38].

Among imaging techniques, abdominal ultrasound is the most widely used in patients with ALD. Ultrasound can detect hepatic steatosis if it affects 30 % of the liver [39, 40]. In severe ALD, Doppler ultrasound can detect the presence of a cirrhotic liver as well as signs of portal hypertension (splenomegaly, changes in portal vein flow, presence of collaterals and ascites). Moreover, it is the technique of choice for the screening of portal vein thrombosis and HCC. More sophisticated techniques such as abdominal CT scan and magnetic resonance imaging (MRI) are more accurate than ultrasonography for evaluating steatosis, but are not cost-effective [41]. They are used for a more precise diagnosis of HCC or when there is suspicion of biliary obstruction. Of note, imaging studies cannot establish alcohol as the specific etiology of a given chronic liver disease.

Noninvasive Diagnosis of ASH and Fibrosis

In the last decade there have been major advances in noninvasive techniques to assess the severity of a liver disease. Because patients with ASH and fibrosis are at high risk for developing cirrhosis, it is important to detect these progressive forms of ALD. Several serum tests developed for the noninvasive assessment of viral hepatitis have also been tested in patients with ALD [42]. They include serum biomarkers and elastography. The AST to platelet ratio index has a limited value in the diagnosis of fibrosis in ALD [43]. More sophisticated serum tests such as FibroTest®, FibrometerA®, Hepascore®, and ELF have been evaluated in patients with ALD [44–47]. FibroTest® is a marker panel composed of alpha-2-macroglobulin, haptoglobin, GGT, ApoA1, and bilirubin, corrected for age and sex [48]. It has high diagnostic accuracy for the detection of significant fibrosis in patients with ALD [44]. Importantly, the ELF test may predict clinical outcomes in patients with chronic liver disease, but its efficacy was not validated in larger cohorts of ALD patients [47]. The AshTest estimates the presence of ASH from the patient's levels of α2-macroglobulin, haptoglobin, ApoA1, total bilirubin, GGT, ALT, and AST [49]. In heavy drinkers, AshTest represents a potential noninvasive marker to estimate of the presence of ASH [49].

The measurement of liver stiffness by transient elastography (Fibroscan®) represents a major advance in the noninvasive assessment of the degree of liver fibrosis including patients with ALD [50, 51]. This device was recently approved by the FDA. In patients with ALD, liver stiffness correlates with the degree of fibrosis [52]. However, inflammation, cholestasis, or liver congestion may affect liver stiffness measurement independently of the degree of fibrosis [53]. Elevated liver stiffness values in patients with ALD and AST serum levels >100 U/L should be interpreted with caution due to the possibility of falsely elevated liver stiffness as a result of superimposed ASH [42]. Moreover, recent alcohol consumption can also elevate liver stiffness, perhaps related to the vasodilatory effects of alcohol [54]. Thus, the correct interpretation of liver stiffness requires a timely abdominal ultrasound and actual transaminase levels and alcohol consumption. Controlled attenuation parameter (CAP) is run on the Fibroscan platform. Preliminary results suggest that CAP is reproducible and quantitative with an AUROC up to 90 % for fatty liver [55].

Liver Biopsy

Liver biopsy is rarely indicated in patients with high suspicion of ALD, though it is the most accurate method to establish the severity of liver injury in ALD [56, 57]. Liver biopsy can be done percutaneously but often requires a transjugular approach in patients with advanced ALD due to coagulopathy. The morphological features of ALD encompass four elementary lesions: macrovesicular steatosis, centrilobular ballooning of hepatocytes, neutrophil infiltrate that predominates in the lobules, and variable degrees of liver fibrosis [58]. Macrovesicular steatosis is the most frequent pattern of alcohol-induced liver injury

[58]. Neutrophil infiltration is typically first seen in zone 3 (perivenular). As the disease progresses, the histologic changes also affect zone 2 and even zone 1 (periportal) hepatocyte. The presence of neutrophils is a hallmark of ASH. Mallory-Denk bodies are eosinophilic accumulations of intracellular protein aggregates within the cytoplasm of hepatocytes. They represent condensations of intracellular "intermediate filaments" or cytokeratins that are normal components of the hepatocyte cytoskeleton [59]. They are not specific to ASH and can be seen in nonalcoholic steatohepatitis (NASH) [60]. Alcoholic fibrosis is typically located in pericentral and perisinusoidal areas and first appears in the zone 3 area [61], and there is underlying cirrhosis in many cases [17, 19]. Patients with ALD can develop a particular pattern of liver fibrosis (chicken wire) due to massive pericellular and perisinusoidal deposition of collagen fibers (Fig. 10.2).

The clear indications of liver biopsy are not established; however, it should be considered in patients with severe AH requiring specific medical therapy and in patients with other coexisting factors contributing to liver disease. Relying on clinical criteria alone carries a 10–50 % risk of misclassifying patients as having or not having AH [62, 63]. Therefore, the recently published EASL Practical Guidelines on Alcoholic Liver Disease recommends a liver biopsy, if available, in patients with suspected severe AH [64]. In patients with the clinical syndrome of AH and any one of the followings factors, liver biopsy is specially recommended: (1) hypotension/massive bleeding at admission, (2) sepsis at admission, (3) suspicion of malignant liver disease based on clinical and/or imaging criteria, (4) uncertain assessment of alcohol drinking history, (5) cocaine use in the last 3 months, and (6) recent use of a potential hepatotoxic substance. Future studies should identify noninvasive markers capable of estimating the presence of AH without the need of a liver biopsy.

Diagnosis of Alcoholic Cirrhosis

Alcoholic cirrhosis can be silent for many years, and therefore it can only be diagnosed if analytical and imaging studies are performed. Often, patients with alcoholic cirrhosis are diagnosed upon the development of a clinical complication. Patients who have decompensated cirrhosis may report jaundice, weakness, peripheral edema, abdominal distension, or symptoms of gastrointestinal bleeding. Cirrhotic patients usually have stigmata of chronic liver disease such as palmar erythema, spider angiomata, and gynecomastia. Asterixis is seen in patients with hepatic encephalopathy. A low number of platelets are indicative

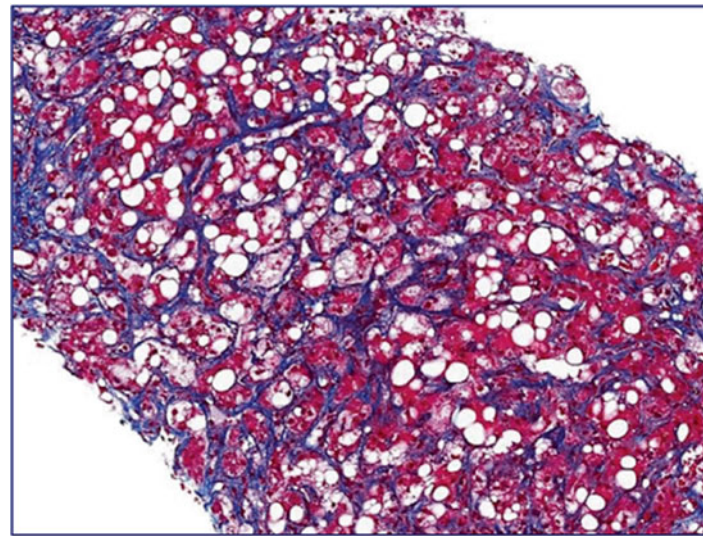

Fig. 10.2 Panlobular fibrosis in a patient with severe alcoholic liver disease showing a typical "chicken wire" pattern

of cirrhosis or advanced fibrosis in chronic liver disease. Thrombocytopenia may result from primary bone marrow hypoplasia or splenic sequestration. Leukopenia and anemia develop later in the disease course [65]. In patients with ALD, anemia is typically megaloblastic. Albumin levels fall and INR increases as the synthetic function of the liver declines.

Specific imaging features indicating the presence of alcoholic cirrhosis include a nodular appearance of the liver, higher volume index of the caudate lobe, frequent visualization of the right posterior hepatic notch, and smaller size of regenerative nodules of the liver [66]. In early cirrhosis without major macroscopic changes, the sensitivity of imaging studies is less than 70 %. Thus, ultrasound findings are useful to confirm the presence of alcoholic cirrhotic livers, but a negative result cannot fully rule out cirrhosis. As described above, noninvasive techniques can be useful to detect the presence of cirrhosis, as well as a liver biopsy in special cases. Of note, noninvasive tests including elastography are not specific enough to detect the presence of esophageal varices, so an upper endoscopy is indicated in all patients with the diagnosis of liver cirrhosis.

Diagnosis of AH

The diagnosis of AH is made on clinical grounds, based on a history of excessive alcohol use with the typical physical exam and laboratory findings. Liver biopsy may be helpful to establish the presence of ASH and has been endorsed in recent clinical practice guidelines [64, 67]. As detailed above, a biopsy is particularly valuable in the setting of atypical clinical characteristics or when the diagnosis remains in doubt. A transjugular route is often preferred due to frequent coexisting ascites and/or coagulopathy. Infections, particularly spontaneous bacterial peritonitis, must be ruled out as they can present with similar clinical findings (abdominal pain, fever, leukocytosis) and because they are a contraindication to specific therapy with corticosteroids. Serologic evaluation for viral hepatitis should be undertaken as well. Imaging with Doppler ultrasound is important to exclude biliary or vascular disorders and to evaluate for coexisting hepatocellular carcinoma. Contrast-enhanced computed tomography or magnetic resonance imaging is often helpful to confirm ultrasound findings.

Recently, we performed a large multicentric study to develop a histological scoring system capable of predicting short-term survival in patients with AH. The resulting alcoholic hepatitis histological score (AHHS) comprises four parameters that are independently associated with patients' survival: fibrosis stage, PMN infiltration, type of bilirubinostasis, and presence of megamitochondria. By combining these parameters in a semiquantitative manner, we were able to stratify patients into low, intermediate, or high risk for death within 90 days [68].

Assessment of Extrahepatic Alcohol-Induced Organ Damage

Patients with ALD often have coexisting dysfunction in extrahepatic organs and may have signs of malnutrition, cardiomyopathy, neuropathies, pancreatic dysfunction, and skeletal muscle wasting (sarcopenia). A nutritional assessment is necessary for all patients with ALD due to the association between alcoholism and nutritional deficiencies such as protein, calories, minerals (e.g., zinc), and vitamins (e.g., vitamin D). Long-term excessive alcohol consumption can also be a cause of cardiomyopathy [69]. A history of heavy and prolonged alcohol intake in addition to the signs and symptoms of heart failure is the basis of a diagnosis of alcoholic cardiomyopathy, which can be confirmed by echocardiography. Chronic alcoholic myopathy is defined by muscle atrophy and weakness predominantly in the proximal muscles and may affect 40–60 % of alcoholics [70]. The association between myopathy and neuropathy in alcoholics might be due to direct effects on ethanol/acetaldehyde and ethanol-mediated oxidative stress on muscle and neurons [71]. Although patients with ALD typically do not have concomitant pancreatic disease, patients with chronic abdominal pain or steatorrhea

should be worked up to rule out chronic pancreatitis. Acute pancreatitis is one of the most severe complications in patients with alcohol use disorder. Due to the progression of pancreatic fibrosis from ethanol, prolonged drinking may lead to the development of chronic pancreatitis [72]. Chronic pancreatitis is common in alcoholics and poses a risk for the development of pancreatic cancer.

Alcohol abuse is commonly associated with an array of neurological disorders. A complete neurological examination is therefore recommended in patients with ALD. The most common type of central nervous system damage from heavy drinking is brain atrophy, which may lead to dementia [73]. Mild cognitive impairment has been reported in 50–80 % of alcoholics and can be difficult to differentiate from chronic encephalopathy [74]. Wernicke's encephalopathy is an acute neurological disorder that is caused by thiamine deficiency and manifests in a clinical triad of encephalopathy, oculomotor dysfunction, and gait ataxia. Prevention of this syndrome relies on thiamine administration, particularly for patients receiving intravenous glucose. Alcoholics often present peripheral polyneuropathy, caused by nutritional vitamin B complex deficiency and the direct toxic effect of acetaldehyde. The pathogenesis of this disorder may involve ethanol-induced lipid peroxidation and defective antioxidant mechanisms within the sciatic nerve [75]. Alcoholic polyneuropathy is a gradually progressive disorder of sensory, motor, and autonomic nerves. Symptoms include numbness, paresthesia, burning dysesthesia, pain, weakness, muscle cramps, and gait ataxia. The prognosis of alcohol-induced neurological manifestations is poor since most of the symptoms do not reverse after prolonged abstinence.

Clinical End Points in Patients with ALD

The most effective therapy for all patients with ALD, regardless of the disease stage, is prolonged abstinence from alcohol. Improved clinical outcomes are observed with abstinence across the spectrum of ALD, from the early to most severe cases [19, 64]. Therefore, prolonged alcohol abstinence is the first and most important clinical end point for patients with ALD. In patients with early asymptomatic phenotypes (fatty liver and ASH with or without mild fibrosis), the clinical end points consist of normalization of laboratory abnormalities and resolution of fibrosis. There are several noninvasive tests to assess the degree of fibrosis; however, none of these tools have been validated in longitudinal studies. As such, there is a clear need to develop noninvasive tools to monitor the response to therapy in patients with early ALD. In contrast, the high short-term mortality of patients with severe ALD, such as decompensated cirrhosis, makes prolonged short-term survival the main clinical end point for these patients. Other important end points include a reduction in the occurrence and severity of clinical complications and the improvement of tests indicative of liver failure (i.e., ABIC or MELD score).

Achieving Prolonged Alcohol Abstinence

Achieving and maintaining alcohol abstinence are the most important treatment goal for patients with ALD, since abstinence improves patient survival and prognosis [76, 77]. Abstinence is also critical for patients with advanced disease who may eventually require liver transplantation, because patients who actively engage in alcohol consumption are not eligible for most programs. The life event of AH should be used to initiate early interventions to achieve abstinence. Referral to addiction specialists or rehabilitation is recommended. *Early referral from hospitalization, as well as the use of a multidisciplinary team including an addiction therapist, increases patient adherence and the number of patients who achieve prolonged abstinence (unpublished observations).*

With initial abstinence achieved, prolonged maintenance becomes the primary goal for

patients with ALD. In addition to addiction counseling and rehabilitation, anticraving drugs may help prevent alcohol relapse. Disulfiram is frequently prescribed for the treatment of alcoholism [78], but is not recommended in patients with advanced ALD due to its potential severe hepatotoxicity [79]. Acamprosate and naltrexone also reduce the withdrawal effects of and the craving for alcohol, but they can also cause hepatotoxicity [80, 81]. Baclofen, a GABA-B receptor agonist, has been found to be effective in the maintenance of abstinence. Importantly, a study involving patients with liver cirrhosis found that a 12-week course of baclofen effectively maintained abstinence by reducing the craving for alcohol without causing hepatotoxicity [82]. This study was recently confirmed [83], suggesting that baclofen is the only fully safe anticraving drug for patients with ALD.

Slowing Disease Progression in Patients with Early Forms of ALD

There are few studies assessing strategies to slow down or even reverse fibrosis progression in patients with ALD. The lack of studies is influenced by intrinsic difficulties in performing clinical trials in patients with an active addition (e.g., poor compliance). Moreover, patients enrolled in clinical trials are likely to reduce their alcohol intake, which can attenuate fibrosis progression. The fact that placebo-treated patients in large trials showed reduction in fibrosis supports this assumption [84]. To date, the only effective therapy to reverse fibrosis in patients with ALD is abstinence from alcohol. Total abstinence from alcohol consumption enhances the clinical outcome of liver disease, this being the most important factor in determining long-term survival in alcohol-related cirrhosis [85]. There are few systematic reports indicating that alcohol abstinence is the main determinant of outcome in patients with compensated ALD [86]. Moreover, isolated reports indicate that alcohol abstinence is followed by fibrosis regression [87]. *Disease progression of patients with ALD is heavily influenced by both genetic and environmental factors.* Prospective studies are needed to uncover the genetic and environmental factors involved in fibrosis resolution.

There are three critical steps needed to develop new antifibrotic therapies for patients with ALD. First, it is important to define the patient population in terms of alcohol consumption. Patients unable to completely stop drinking but likely to be compliant during the clinical trial should be identified. Second, clinical trials should incorporate noninvasive markers of liver fibrosis to monitor the response to therapy, since performing paired biopsies in placebo-treated patients is not ethical. And, third, antifibrotic drugs that target key pathogenic drivers in ALD should be selected. The ideal antifibrotic drug should be relatively cheap, well tolerated over prolonged periods, and not associated with hepatotoxicity or HCC development. Large well-designed clinical trials with this and other targeted therapies should be tested in patients with ALD.

Improving Survival in Patients with Alcoholic Cirrhosis

Once liver cirrhosis is established, abstinent patients may present a slower disease progression than those actively consuming alcohol. Moreover, patients with persisting alcohol intake may develop some degree of ASH that may lead to a higher risk of decompensation (conditions such as ascites, hepatic encephalopathy, variceal bleeding, or renal dysfunction) or to the development of HCC. Management of alcoholic cirrhosis focuses on alcohol abstinence, nutritional therapy rich in calories and proteins [88], and prophylaxis of cirrhosis complications. Regarding clinical decompensations, there is no evidence supporting different management strategies in alcoholic cirrhosis compared to other causes of cirrhosis other than the encouragement of alcohol intake cessation.

Improving Survival in Patients with AH

Alcoholic hepatitis carries high short-term mortality (around 30–50 % at 3 months). Treatment of patients with AH has not substantially improved in the last decades. Therefore, the main goal in the management of AH is to improve short-term mortality. Several prognostic models have been developed to identify patients with AH who are at high risk of death within 1–3 months of their hospitalization. The most widely used is Maddrey's discriminant function (DF) [89]. The DF value ≥32 is indicative of a high risk of short-term mortality (35 % at 1 month) and is the basis for patient selection for specific therapy with corticosteroids. Additional predictive models include the Model for End-Stage Liver Disease (MELD), the Glasgow AH score, the ABIC score, and the Lille model (www.lillemodel.com) [90–92]. Finally, histological assessment using the recently developed AHHS is able to stratify patients into low, intermediate, or high risk for death within 90 days [68].

General measures for the management and treatment of complications related to AH have not been shown to improve survival, but they should be considered as part of the standard of care. Patients with severe AH may require admission to an intensive care unit. The airway should be protected in patients with acute alcoholic intoxication or an advanced degree of hepatic encephalopathy. Benzodiazepines are generally contraindicated in these patients, but might be necessary in the case of severe alcohol withdrawal. There is a potential risk of Wernicke's encephalopathy among alcoholic and malnourished patients; thus, the administration of vitamin B complex is recommended. Nutritional support improves liver function, and short-term follow-up studies suggest that improved nutrition might improve survival times and histological findings in patients with AH [88, 93, 94]. In patients without encephalopathy, oral supplements and/or feeding through a nasogastric tube is preferred over total parenteral nutrition in order to avoid gram-positive bacterial infections.

Corticosteroids are widely used as the first-line therapy and improve short-term survival in patients with severe AH [95]. The response to prednisolone can be assessed based on the change in bilirubin after 1 week of therapy and quantified using the Lille score [96]. For those with a poor response as indicated by a Lille score ≥0.45, stopping therapy can be considered, as there is likely no benefit to continuing steroids in this setting. Pentoxifylline reduced mortality in patients with severe AH [97] and is typically reserved as a second-line agent for patients with contraindications to corticosteroid therapy (i.e., uncontrolled infection, GI bleeding). However, in a large randomized controlled trial (STOPAH) that included more than 1000 patients, presented during the 2014 AASLD meeting, pentoxifylline was not better than placebo in terms of short-term mortality [98].

Unfortunately, there are no available rescue therapies for patients not responding to standard therapy. The combination of prednisolone and pentoxifylline offers no benefit [99]. A recent randomized trial showed that the combination of N-acetylcysteine with prednisolone reduced 1-month mortality (8 % vs. 24 %) and the incidence of hepatorenal syndrome and infection [100]. The favorable safety profile of N-acetylcysteine makes it a potential option, in combination with corticosteroids, for patients with severe disease. There is a clear need to develop novel targeted therapies for patients not responding to existing drugs.

Acute kidney injury (AKI) is one of the common complications of AH [101]. The presence of AKI, often due to superimposed hepatorenal syndrome, is associated with a bad prognosis [17, 101]. As such, AKI prevention and early treatment are paramount. Volume expansion with albumin or crystalloid should be a priority in the early therapy of AH to prevent pre-renal azotemia and acute tubular necrosis. Interestingly, patients with systemic inflammatory response are at high risk of developing AKI.

Prevention and Early Treatment of Infections

Infections are also common in patients with AH [102]. All patients with clinical AH should be systematically screened for infection with chest x-rays and cultures of blood, urine, and ascites. Particularly, spontaneous bacterial peritonitis must be ruled out as it can present with similar clinical findings (abdominal pain, fever, leukocytosis) and because it is a contraindication to specific therapy with corticosteroids. Because patients with AH are predisposed to develop severe infections, empiric antibiotics may be administered if there is a high suspicion of infection. A clinical trial on the role of prophylactic antibiotics in patients with AH is currently ongoing (https://clinicaltrials.gov).

End Points in Ongoing Clinical Trials

Most ongoing clinical trials are being performed in patients with AH (Table 10.2). In most studies, the diagnosis is confirmed through histological analysis. The main primary end points are related to survival (1–6-month survival), although some studies chose improvement of scoring systems that reflect liver function (i.e., MELD or bilirubin) as primary end point. These surrogate end points are less powerful but allow a more favorable power calculation analysis with a lower number of patients needed to achieve positive results. Secondary end points are more heterogeneous and include development of infections, MELD score changes, occurrence of decompensations or infections, etc.

Few ongoing studies are evaluating therapeutic interventions in patients with other forms of ALD (Table 10.2 in bold). Many of the studies do not require a histological confirmation, and they compare a novel drug with placebo. Regarding the end points, some of them consider improvement in liver function tests, and few studies aim to improve histology. Importantly, only few studies consider abstinence as an end point. There is a clear need to perform more clinical trials in patients with early forms of ALD and to better define inclusion criteria, monitoring of response, and the most accurate primary and secondary end points.

Conclusions and Prospects for the Future

Alcohol consumption is a leading cause of global morbidity and mortality, with much of its negative impact as a result of ALD. The diagnosis of ALD is based on medical history, physical exam, and noninvasive tests, and liver biopsy is only recommended in severe cases such as AH. Despite some important advances in our understanding of the pathogenesis and clinical characteristics of ALD, there have been no significant advances in therapy in the last 40 years. The mainstream of therapy for any patients with ALD, regardless of the disease stage, is prolonged alcohol abstinence. Abstinence is associated with improved clinical outcomes across the spectrum of ALD.

Clinical end points depend on the stage of ALD. In compensated patients, the end points consist of normalization of abnormal lab tests and reduction of liver fibrosis. These end points can be monitored noninvasively. In patients with AH and decompensated cirrhosis, the clinical end points are survival and compensation of the liver disease. In the long-term, it is possible that early alcoholic cirrhosis can reverse to a nearly normal liver. The genetic and environmental factors that influence disease reversibility after prolonged abstinence are unknown and deserve prospective studies. Recent translational work using human liver tissue has been informative in identifying some potential therapeutic targets for severe ALD. However, translation of these findings into novel therapies has been lacking. Additional detailed studies of these potential targets in humans and animal models are urgently needed to improve outcomes in this patient population.

Table 10.2 Ongoing clinical trials for patients with alcoholic hepatitis and other forms of alcoholic liver diseases (in bold)

Biopsy	Therapeutic approach	Control group	Primary end point	Secondary end point	Status	ClinicalTrials.gov identifier
Yes	NAC	Placebo	6-month survival	Infection	Completed	NCT00962442
No	Probiotics	Placebo	MELD at 30 days		Recruiting	NCT01922895
No	Metadoxine + PRED or PTX	PRED or PTX	30-day survival	3- and 6-month survival	Active	NCT02161653
Yes	Rifaximin + PRED for 4 weeks		Bacterial infections	Decompensation	Recruiting	NCT02116556
No	G-CSF (steroid nonresponder)	Placebo	1- and 3-month survival	Child score MELD score	Recruiting	NCT01820208
Yes	PRED + NAC	PRED	1-, 3-, and 6-month survival	Bilirubin	Completed	NCT00863785
Option	Obeticholic acid	Placebo	MELD at 6 weeks	Side effect	Active	NCT02039219
Yes	Mycophenolate mofetil, rilonacept	PRED	1- and 4-week survival	MELD score DF score	Active	NCT01903798
No	PTX	PRED	1-month survival		Recruiting	NCT01455337
Yes	Intensive nutrition	Standard therapy	6-month survival	1-month survival	Complete	NCT01801332
Yes	Anakinra, PTX, zinc	PRED	6-month survival	MELD score	Recruiting	NCT01809132
No	PTX		1-month survival		Completed	NCT00205049
Option	IDN-6556 (patients with contraindication to steroid)	Placebo	1-month survival		Recruiting	NCT01912404
Option	SAMe	Phosphatidyl choline	Bilirubin		Recruiting	NCT02024295
Yes	PTX	Placebo	6-month survival		Completed	NCT01214226
Yes	NAC + PRED	PRED	1-, 3-, and 6-month survival	Bilirubin	Recruiting	NCT00863785
Yes	NAC	Placebo	6-month survival	Infection	Completed	NCT00962442
No	Hyperimmune bovine colostrum	Placebo	Endotoxin level		Active	NCT01968382
No	**MG**	**Placebo**	**Liver enzymes**		**Recruiting**	**NCT02019056**
Yes	**Candesartan + UDCA**	**UDCA**	**Histology at 6 months**	**Safety**	**Completed**	**NCT00990639**
Yes	**SAMe**	**Placebo**	**Homocysteine, SAMe**		**Recruiting**	**NCT00851981**
No	**Probiotics**	**Placebo**	**Liver enzymes**		**Active**	**NCT01501162**
No	**SAMe**	**Placebo**	**Liver enzymes**	**SAMe level**	**Completed**	**NCT00573313**
No	**Baclofen**	**Placebo**	**Alcohol consumption**		**Recruiting**	**NCT01711125**
No	**Polymeric nutritional supplements**	**Standard therapy**	**1-year survival**		**Active**	**NCT02140294**
No	**Metadoxine**	**Placebo**	**Abstinence**	**Liver enzyme**	**Recruiting**	**NCT01504295**
No	**Zinc**	**Placebo**	**Clinical status**		**Recruiting**	**NCT02072746**
No	**Baclofen**	**Placebo**	**Abstinence**		**Recruiting**	**NCT01455337**
Option	**Losartan**	**Placebo**	**Survival**			**NCT00239096**
No	**Protandim**	**Placebo**	**Capillary function**		**Recruiting**	**NCT00936000**

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11 Diagnostic Considerations and Clinical End Points for Nonalcoholic Steatohepatitis

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Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NAFL	Nonalcoholic fatty liver
HVPG	Hepatic venous pressure gradient
MELD	Model for End-Stage Liver Disease
T2DM	Type 2 diabetes mellitus
MetS	Metabolic syndrome
FDA	Food and Drug Administration
AASLD	American Association for the Study of Liver Disease

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in North America and is rapidly emerging as a leading cause of liver-related morbidity and mortality [1, 2]. There are two major clinical-histological phenotypes of NAFLD: (a) nonalcoholic fatty liver (NAFL) and (b) nonalcoholic steatohepatitis (NASH) [3]. It is estimated that about 30 % of the adult population and at least 10 % of children in the United States have NAFLD [4–6]. It is further estimated that 20–25 % of individuals with NAFLD have NASH [3, 7]. NAFLD, especially NASH, has been associated with increased morbidity and mortality from cardiovascular-, cancer-, and liver-related causes [8–11]. NAFLD is also increasingly identified as a comorbidity in hospitalized patients [12]. At a time when hepatitis C is declining as an indication for liver transplantation nationally, NASH is rising as an indication for liver transplant and is projected to become the leading indication for liver transplantation over the next decade [13]. Given the growing contribution of NASH to the burden of end-stage liver disease, it is now a health-care priority to develop both diagnostic and therapeutic strategies to ultimately improve the health of this population.

There are three principal questions that need evaluation in those with suspected or known NASH. First, it should be ascertained if NAFLD is present and then if it warrants intervention. Once therapy is started, there is a need to evaluate whether the drug is working or not. We approach subjects with NAFLD using this relatively simple approach.

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Diagnosing the Presence of NAFLD

When to Suspect NAFLD

Most subjects with NAFLD are asymptomatic. In such cases, the diagnosis is often made when they are discovered to have either abnormal liver enzymes or features of a fatty liver on an imaging study performed for unrelated reasons. There are also others who are diagnosed because the liver looked unusual during abdominal surgery. There are also some individuals who are diagnosed because of persistent hepatomegaly. It is however important to remember that only a very small minority of subjects with NAFLD have been diagnosed and the great majority of afflicted individuals are currently undiagnosed.

Symptoms Associated with NAFLD When symptoms occur, the most common symptoms that bring NAFLD patients to clinical attention include malaise, fatigue, and right upper quadrant discomfort. The discomfort is often described as a nondescript vague discomfort rather than pain. Such symptoms often antedate the diagnosis of NAFLD in a third of patients.

Fatigue is often present in patients with NAFLD and is often the most debilitating aspect of the condition for the individual subject. In this author's experience, fatigue is the most common symptom in this patient population. Several clinical variations of the fatigue syndrome can be recognized. Most commonly, subjects complain of chronic malaise and a sensation of severe lethargy and sleepiness during the day. This is associated with disturbed sleep patterns, and patients often wake up feeling tired. Many subjects have associated sleep disorders and, upon direct questioning, will acknowledge that they snore. The spouses of affected individuals often report that the patients snore and are very restless at night. There is also a close interrelationship between disordered sleep and insulin resistance. The severity of fatigue does not correlate with the grade or stage of steatohepatitis. The relationship of other abnormalities in sleep disorders and insulin resistance remains poorly defined.

Other subjects wake up relatively rested but tire easily. This pattern is more often associated with those with more advanced disease. Some patients have mixed patterns of fatigue which are often difficult to evaluate and manage. Approximately 25 % of subjects with NAFLD also carry a diagnosis of chronic fatigue syndrome [14]. Some patients complain of aching soreness in their muscles along with fatigue, and about 20 % of all subjects suffer from a chronic pain disorder [15].

There are also a number of common conditions associated with NAFLD. The relationship of NAFLD to underlying obesity, type 2 diabetes, hypertension, and dyslipidemia is well known. Many subjects also have anxiety disorders or depression. The contribution of underlying depression and antidepressants to weight gain and NAFLD is unclear.

Physical Examination Obesity is the most common physical finding and is present from 50 to 90 % of subjects [3]. About two thirds of subjects with NAFLD also have other features of the metabolic syndrome. Hepatomegaly is the most common liver-related physical finding in subjects with NAFLD [15]. A minority of subjects have stigmata of chronic liver disease such as spider angiomata or palmar erythema. Jaundice and features of portal hypertension, i.e., ascites, variceal hemorrhage, etc., are seen in a small minority of subjects who present with advanced liver disease. Occasionally, NASH can present as subacute liver failure [16].

Whether depression and anxiety disorders are unrelated or somehow related to NAFLD remains to be evaluated. Also, the prevalence of NAFLD in the general population of subjects with chronic pain or fatigue is unknown. One should however be aware that NAFLD may also be present in subjects with chronic fatigue, fibromyalgia, and depression.

How to Confirm the Presence of NAFLD

This is largely based on imaging studies. While imaging studies can identify the presence of steatosis, they are unable to distinguish between steatosis and steatohepatitis. Liver biopsy remains

the gold standard for the diagnosis of steatohepatitis. It is however limited by sampling variability, its invasive nature, associated morbidity which can be severe, and rare mortality. Its use simply to document the presence of excess fat in the liver has largely been replaced by noninvasive methods of testing.

Two-dimensional sonography, computerized tomography (CT), and magnetic resonance imaging (MRI) are the most studied methods that are also available at most medical centers. The sonographic features of NAFLD include increased hepatic parenchymal echotexture and vascular blurring [17]. These findings are however also seen in those with any form of chronic liver disease, and although sensitive (85–95 %), they are nonspecific (positive predictive value 62 %). Also, the ability to detect fatty liver by sonography drops off markedly once the degree of hepatic steatosis decreases to 30 % or less [18, 19].

CT imaging of the liver provides a more specific method for the noninvasive diagnosis of NAFLD. Hepatic steatosis decreases the CT attenuation of the liver. When the hepatic parenchymal attenuation is 10 or more Hounsfield units lower than the spleen on a non-contrast-enhanced scan, a diagnosis of hepatic steatosis can be made. When intravenous contrast is administered, the hepatic enhancement lags behind the spleen, and the liver-to-spleen attenuation differential exceeds 20 Hounsfield units [20]. While these features allow hepatic steatosis to be defined with a 76 % positive predictive value [21], they do not permit distinction between fatty liver and steatohepatitis. Also, the diagnostic sensitivity of the test depends on the severity of the steatosis and falls off when the steatosis is mild. CT imaging also does not provide any information on the stage of fibrosis in the liver unless features of portal hypertension are present. This only occurs in the presence of cirrhosis of the liver. Finally, it is worth remembering that CT scans are substantially more expensive than sonography.

Magnetic resonance imaging (MRI) is even more sensitive than a CT scan for the assessment of hepatic steatosis. MRI techniques have been refined to provide a highly reproducible and validated measure of hepatic triglyceride content [22, 23]. Unfortunately, MRI also has many of the same limitations of CT imaging noted above. Recent development of proton density fat fraction methods provides data that are comparable to that with spectroscopy but interrogate the entire liver section [23] However, it is important to note that none of these methods can diagnose steatohepatitis or accurately assess the stage of the disease.

Is Intervention Warranted?

The ultimate goal of therapy is to prevent liver and extrahepatic morbidity and mortality associated with NAFLD. There is an excess of cardiovascular-, liver-, and cancer-related mortality in subjects with NAFLD [8]. 15–20 % of subjects with NASH progress to cirrhosis, whereas only 2–3 % of subjects with NAFL demonstrate disease progression. The 10-year outcomes of those with NAFL alone are quite good from a liver perspective, and in such cases, the focus remains on lifestyle interventions and the management of cardiovascular risk.

Liver-related mortality is mainly related to the development of cirrhosis. It is therefore important to know if a subject has already progressed to cirrhosis or has advanced fibrosis (bridging fibrosis). Also, in subjects with earlier-stage disease, it is important to identify those at risk for disease progression to cirrhosis and prioritize them for treatment. Based on these principles, two broad approaches are taken.

First, it is important to evaluate if there is underlying cirrhosis or bridging fibrosis. Both are associated with a significant worsening of clinical stage and warrant treatment. While a liver biopsy was required in all cases in the past, the development of noninvasive methods has markedly reduced the need for biopsy to answer this question. Several such markers have been developed.

The FIB4 is based on the age, AST, ALT, and platelet count [24]. It has been shown to correlate with fibrosis stage and identifies bridging fibrosis or cirrhosis with a ROC of 0.8 in a large cohort of highly characterized subjects with NASH. The NAFLD fibrosis score incorporates these

parameters and also includes measures of glycemia, obesity, and albumin. The NAFLD fibrosis score has also been shown to correlate with the fibrosis stage with similar predictive values as the FIB4 test [25]. The AST to platelet ratio also differentiates advanced from modest fibrosis. All of these tests have further been shown to correlate with outcomes [26].

Transient elastography is a technique where a shear wave is propagated through the liver and the propagation pattern used to determine the elasticity of the liver. The greater the amount of fibrosis present, the more stiff the liver becomes, and the wave is propagated more rapidly. Several types of elastography-based methods are currently available and approved by the FDA for the measurement of liver stiffness. These include vibration-based methods such as the fibroscan and ARFI as well as MR-based elastography [27]. Fibroscan can be performed at the point of care but may not be accurate when there is severe obesity. A special probe (XL) has been developed to overcome this limitation when it is performed with the conventional M probe. Transient elastography results may be confounded by hepatic congestion following a meal or from congestive heart failure. It is also confounded by the presence of severe cholestasis [28]. A value less than 8 kpascals correlates with no fibrosis, while values over 20 kpascals have been associated with cirrhosis and clinically significant portal hypertension [29]. Recent innovations allow three-dimensional assessment of elastography by MRI and along with its ability to quantify steatosis are becoming a popular tool for clinical trials. Similarly, a continuous attenuation parameter (CAP) program has been developed for a semi-quantitative assessment of hepatic steatosis [30]. The use of fibroscan and MR-based assessment methods in routine clinical practice is evolving and likely to change over the next few years.

Next, in those who do not appear to have advanced stage disease, it is necessary to determine if they are at risk for the development of advanced disease. The presence of multiple features of the metabolic syndrome especially type 2 diabetes, elevated ALT, increasing age (>50 year), and progressive weight gain have all been linked to increased risk of disease progression. Subjects with these risk factors who do not have features of advanced fibrosis are targeted for a liver biopsy to confirm the presence of steatohepatitis. Steatohepatitis is defined either as a gestalt based on the pattern of steatosis, inflammation, and ballooning in the liver or from the presence of steatosis, inflammation, and either ballooning or pericellular fibrosis [31, 32]. The latter has been linked to disease outcomes. Those with steatohepatitis without any fibrosis have an excellent short- and intermediate-term prognosis. On the other hand, those with some degree of fibrosis and those with portal inflammation have been shown to progress more rapidly. Thus, those with steatohepatitis with some degree of fibrosis also constitute an indication for therapeutic intervention beyond lifestyle changes.

Assessing the Outcomes of Therapy

Currently, there is scientific evidence from phase 2b studies on the efficacy of vitamin E, insulin sensitizers (metformin and thiazolidinediones), and the FXR agonist obeticholic acid for the treatment of NASH. However, there are no approved therapies for NASH, and all pharmacological treatment is considered an "off-label" use of the specific agents. In those where specific anti-NASH therapy is used, it is imperative to determine whether the individual is responding. Also, patients must be evaluated for potential "off-target" toxicities. Given the increased cardiovascular risk in subjects with NASH, it is particularly important to keep track of their atherogenic risk profile during treatment. This is underscored by potential concerns about the long-term safety of vitamin E, thiazolidinediones, and obeticholic acid with respect to cardiovascular effects. While increased cancer-related mortality has been reported in NASH, there are no systematic efforts ongoing to better understand this and to develop preventive strategies against it. There is an increased risk of a variety of cancers in those with NASH. In the absence of any scientific data to guide management, we recommend that current practice guidelines for common

cancers such as breast cancer, uterine and cervical cancer, prostate cancer, and colorectal cancer be followed. In those known to have Barrett's esophagus due obesity-related gastroesophageal reflux should be followed according to clinical best practices.

Assessing Liver-Related End Points in a Patient Being Treated for NASH

The liver-related response to treatment can be considered both in terms of drug-agnostic criteria for response and drug-specific criteria. A critical component of the development process for NAFLD is the demonstration of benefit with respect to clinically meaningful outcomes and the development/validation of surrogates that predict an alteration in risk of developing such outcomes. Clinically meaningful outcomes are broadly defined as those that are related to how an affected individual feels, functions, or survives. It is also germane to consider outcomes from the patient's perspective and create a hierarchy of patient-centered outcomes of interest to guide diagnostic and therapeutic development for NASH.

Clinically Meaningful Outcomes in NASH (Table 11.1)

Outcomes and End Points Related to How a Patient with NASH Feels

Both adults and children with NASH have poorer quality of life scores compared to healthy controls [33–35]. These include total, physical, and psychological measures of symptoms and quality of life scores. Up to 50–70 % of subjects with NAFLD have been reported to have depression and/or anxiety [36]. Subjects with NASH have worse scores than those with NAFL, and, as expected, those with cirrhosis have the poorest scores compared to those with earlier stages of the disease. Those with NAFLD also have both poorer physical and mental health scores compared to US populations with or without chronic illness [34].

Given the frequency of these symptoms both in those with early-stage and advanced stage disease and their impact on the lives of affected patients, there is a need to incorporate validated tools to assess symptoms and physical and mental health in the development plans for drugs particularly in phase 2b–4 studies. This will be particularly important to establish that no unexpected "off-target" behavioral adverse event occur with a given treatment.

Table 11.1 Clinically meaningful outcomes

End points related to how a patient feels
– Quality of life
End points related to how a patient functions
– Days from work lost
– Ability to carry on day-to-day activities
End points related to how a patient survives
– Mortality
– Liver-related outcomes: variceal hemorrhage, hepatocellular cancer, ascites, encephalopathy

Outcomes and End Points Related to How a Patient Functions

Most subjects with NAFLD including NASH are able to function and maintain a job and manage day-to-day activities. There is a paucity of data regarding disability in subjects with NAFLD. In a study of subjects with varying types of chronic liver [35], obesity and NAFLD were associated with poorer physical activity compared to other chronic liver diseases and lower levels of body weight. There is a need for additional research to identify the frequency and prevalence of physical and mental disability in those with NAFLD correcting for confounders such as obesity, diabetes and its complications, etc.

Regardless of etiology, cirrhosis leads to a progressive impairment in function eventually impairing the ability to manage daily living activities [37, 38]. This negatively impacts patients and their caregivers and their ability to remain fully employed forcing many families into bankruptcy and even homelessness [37]. These data have at least two implications for drug development for NASH: in studies targeting advanced stage disease, it will be valuable to capture the functional status of individual subjects and the impact of functional impairment on caregivers and health-care resource utilization [1], and these data once again underscore the need for

developing therapeutics before advanced disease develops where therapeutics may or may not fully reverse functional impairment due to the underlying liver disease [2].

Clinical Outcomes and End Points Related to How a Patient Survives

All-cause mortality has long been held as the ultimately most important outcome and thus a key end point in therapy for many chronic diseases that directly cause death. There is no question that this remains an extremely important outcome for NAFLD as well. NASH is also associated with increased all-cause mortality [9, 39, 40]. There are however numerous reasons why this is an impractical end point for clinical trials for NASH, the aggressive form of NAFLD.

Like other chronic liver diseases, NASH progresses slowly to cirrhosis, the principal driver of liver-related outcomes, over 10–20 years [39, 41]. Approximately 15–20 % of subjects progress to cirrhosis in this time frame, and once cirrhosis develops, decompensation and mortality rates are about 4 % annually, and HCC incidence increases [42, 43]. Demonstration of an improvement in mortality rates (all cause or liver related) will therefore require a large number of subjects followed over 10 years or more. The costs and logistics of such an endeavor are prohibitive and make all-cause or liver-related mortality impractical end points for drug development and approval. The only potential exception may be in studies of specific populations of subjects with NASH and established cirrhosis where these end points are more imminent. Mortality should be tracked in the context of phase 4 post-marketing studies.

There are several other key clinical end points that are particularly relevant for studies in those with NASH and advanced disease. These include the rates of hospitalization, unscheduled clinic and emergency room visits, tests performed, and overall health-care resource utilization as well as time away from work. These are particularly well suited as secondary end points which together with a primary end point that directly or indirectly measures a change in health status that impacts the clinically meaningful outcomes described above will provide a comprehensive picture of the benefits of a given intervention.

Surrogate End Points and Their Use in the Assessment of Treatment Outcomes (Table 11.2)

The challenges surrounding the use of "hard end points" such as mortality have made these a barrier to the development of therapeutics against NASH. It is however permissible to use surrogate

Table 11.2 End points related to survival

End point	Comment	Utility
Death	Strongest end point but sample size and study duration will need to be very large	Impractical
MELD score	Score of 14 identifies a point above which in the absence of transplant survival declines	Objective[a] Validated
Two-point CTP	Transition from Child A to B is clearly associated with poorer survival	Objective-subjective Validated Suffer from ceiling and floor effects
HVPG	Tracks risks of complications and progression	Objective[a] Validated
Composite: Ascites Variceal bleeding Encephalopathy HCC	• Strongly associated with mortality • Quantifiable • Rates of development in controls are known	Objective-subjective[a] Validated
Quantitative liver function tests	Quantitative	Need more validation

[a]May serve as a primary end point for trials in subjects with NASH and advanced fibrosis

end points that have been established and validated as measures of health status that are linked to alterations in the risk of the major clinical outcomes discussed above.

Surrogate end points can be broadly classified in two categories: reasonably accepted, those that are likely to predict clinical benefit [1], and generally accepted—surrogate is established to predict clinical benefit [2]. Using surrogates that the FDA accepts as a valid measure of clinical outcomes/benefit, full approval for an agent can be obtained via the "regular pathway" by pivotal trials using such surrogates as the primary end point.

Surrogate End Points for Early-Stage NASH

The long duration over which NASH evolves before patients experience outcomes creates a major challenge in the design and logistics of clinical trials for those with NASH and early-stage disease. This does not however lessen the need for the development of therapeutics for such individuals given the growing contribution of progressive NASH to the burden of chronic liver disease. It is therefore imperative to evaluate end points that can be achieved within 1–4 years and are reliable surrogates for meaningful outcomes. Moreover, these end points should reflect changes in the disease process and be biologically plausible.

End Points for Early Phase (1–2a) Trials for NASH The principal objectives of early phase trials are to assess safety and to obtain a signal for efficacy that will guide decision making about further development for a given drug. The end points for such trials should include traditional end points for safety including data on potential hepatotoxicity. Efficacy-related end points may be those that relate to proof of mechanism or clinical efficacy. Hepatic triglyceride quantification, liver enzymes, and CK18 are biologically plausible markers of improvement and are also objective, measurable, and sensitive to change. Resolution of steatohepatitis almost never occurs without a decrease in hepatic steatosis. Serum CK18 levels, reflective of apoptotic activity, have been shown to correspond robustly with the improvement in liver histology in two phase 2b clinical trials of NASH in adults and in children, respectively, with a predictive area under the curve over 0.9.

Histology-Based End Points The best short-term (1–2 years) end points that track the progression of NASH are currently based on liver histology. It is known that steatohepatitis, not isolated fatty liver, is associated with a substantial increase in the long-term risk of developing cirrhosis and liver-related outcomes [39, 41]. Logic dictates that reversal of steatohepatitis should therefore be related to a decrease in the risk of developing advanced fibrosis. Recently, it has been demonstrated in a clinical trial of vitamin E for NASH that those who resolved their steatohepatitis had improvement in fibrosis. Reversal of steatohepatitis is thus not only closely linked to the biology of the disease but also has been shown to reduce progression to advanced fibrosis and actually cause disease regression. It therefore meets the criteria for a reasonable short-term end point that is suitable for phase 2b or as a surrogate "likely to predict clinical outcomes" for phase 3 trials for NASH. Given that steatosis and inflammation can decrease with the development of advanced fibrosis, reversal of steatohepatitis should be accompanied by lack of progression to advanced fibrosis (stage 3 or 4) as the end point to be measured in such trials. This can also be used in routine clinical practice. The optimal duration before a liver biopsy is needed to determine histological response is uncertain. In general a biopsy may be considered after 12 months of treatment.

A decrease in the NAFLD activity score (NAS) has also been used as an end point in clinical trials. The use of this end point is limited by a lack of data describing the relationship between changes in NAS and either progression to advanced fibrosis or clinical outcomes. There is currently an urgent need to generate such data to validate the use of the NAS as a legitimate way to assess NASH and the response to therapy. The best way to generate this data is to incorporate a change in NAS as a secondary end point in randomized clinical trials.

Development of Advanced Fibrosis/Cirrhosis as an Intermediate-Term End Point There is little controversy within academia and clinicians that the development of cirrhosis represents a significant worsening of health status and thus a clinically meaningful outcome. The development of bridging fibrosis and cirrhosis represents a histological continuum and is often considered together to represent those with advanced disease. Subjects with cirrhosis have poorer quality of life, symptom, and mental/physical health scores compared to early-stage disease [35, 37]. Cirrhosis impairs the functionality of the individual over time, and the eventual onset of complications leads to death at a well-defined rate of 4–5 % annually [40, 42, 43]. It also increases the rates of hospitalization and overall health-care resource utilization. It therefore meets all three criteria (feel, function, and survive) for a clinically meaningful outcome.

Surrogate End Points for Those with Advanced Disease

The development of an adverse liver-related clinical outcome is generally accepted to predict mortality. There are also several surrogates which are considered "likely to predict" mortality risk in subjects with cirrhosis, e.g., the Model for End-Stage Liver Disease (MELD) score, Child-Pugh-Turcotte (CPT) score, and the hepatic venous pressure gradient (HVPG).

Liver-Related Outcome End Point Liver-related mortality in all chronic liver diseases including NASH is closely linked to the development of hepatocellular cancer, ascites and related complications, variceal hemorrhage, hepatic encephalopathy, and eventually acute-on-chronic liver failure usually due to sepsis [40, 43]. It is now well established that the development of any one of these complications heralds an immediate deterioration in health status (i.e., clinical decompensation) and an increase in mortality risk [44, 45]. A composite liver-related outcome end point can be developed, that is, is measurable, objective, and directly related to mortality. The rates of development of these outcomes in those with compensated cirrhosis are well known [44, 45], and specific data for NASH-related cirrhosis are also known [43]. This is best suited as an end point for those with NASH and compensated cirrhosis. Even in this population, the rates of development of these outcomes will require large sample sizes or ways to enrich the population to increase the likelihood of demonstrating differences between placebo and active treatment. For individual subjects, current practice guidelines for the management of compensated cirrhosis including vaccination, endoscopy, assessment for hepatocellular cancer, and maintenance of nutritional status should be followed.

Child-Pugh-Turcotte Score The Child-Pugh-Turcotte score was originally developed as a tool to evaluate mortality risk following portacaval shunt surgery in those with cirrhosis [46]. Over the last three decades, it has been shown to be robustly associated with intermediate-term mortality (1–5 years). Progression from Child class A to class B robustly measures worsening of a given patient's health status and an increase in mortality risk [47]. It however suffers from a ceiling as well as floor effect of which the latter is relevant for the development of drugs for NASH. It also has a subjective element which led to its abandonment as a way to allocate organs for liver transplantation. Despite these limitations, it remains a time-tested way to assess 1–5-year mortality risk in subjects with advanced liver disease. Progression from Child class A to B or a two-point worsening of the CPT score may be considered as a failure of treatment in patients with NASH and cirrhosis receiving anti-NASH treatment.

MELD Score The MELD score is one of the best predictors of short-term (3-month) mortality risk in those with cirrhosis. It has a greater dynamic range compared to the CPT score and is sensitive to change. All of these attributes led to its adoption to guide organ allocation for liver transplantation [48, 49]. MELD is objective, easy to measure, widely available, and backed up by a very large body of evidence as a rigorous surrogate for mortality risk.

The MELD score is not a good surrogate for outcomes in early-stage NASH, and its use

should be restricted primarily to the treatment of those with NASH and advanced fibrosis (bridging fibrosis or cirrhosis). It has been shown that the benefits of liver transplant start becoming apparent once the MELD scores exceed 14 [50, 51]. Consequently, a MELD score > 14 is often considered a minimal listing threshold for liver transplantation. While it is understood that many other factors also determine if a given patient is listed for transplant, all other things being equal, a MELD of 14 or higher represents a need for liver transplant and therefore is justifiable as a surrogate end point that meets the criteria for a valid surrogate, i.e., strong relationship to mortality and outcomes, objective, easy to measure, sensitive, and widely available.

Hepatic Venous Pressure Gradient (HVPG) The HVPG measures the difference between the wedged hepatic venous pressure and the free hepatic venous pressure. It is related to outcomes, measurable, objective, and sensitive to change. The methodology for its measurement is well established, and there is a large body of literature to support its concordance with liver-related outcomes [52, 53, 54]. Cirrhosis-related complications heralding clinical decompensation occur largely above a threshold HVPG of 10 mmHg which forms the basis for this cutoff to represent clinically significant portal hypertension [55, 56]. The proportions of subjects developing a HVPG > 10 mmHg may also serve as a surrogate primary end point in trials of therapeutics for those with NASH and advanced fibrosis assuming they have lower HVPG at entry into the trial. This end point is best suited for those with advanced fibrosis or cirrhosis. Its role in routine clinical practice remains to be determined.

Quantitative Liver Function Tests There has been considerable interest in the development of such tests as a marker of overall hepatic wellness status and functional reserve. Numerous such tests are in various phases of development. One such test that has been validated both with liver histology and outcomes is the dual oral and intravenous cholate administration test [57]. It is important that any such test be appropriately validated to predict liver-related clinically meaningful outcomes prior to its use as a primary surrogate end point in clinical trials.

Drug-Specific Assessment of Response

In subjects receiving vitamin E for NASH, weight loss and a reduction in liver enzymes have been related to histological improvement. Conversely, weight gain along with persistent elevation of liver enzymes has been associated with lack of histological improvement. There are however no clinical paradigms associated with histological response with thiazolidinedione or with obeticholic acid. There is currently an urgent need to develop noninvasive methods to assess whether a given patient is responding to therapy within 4–12 weeks of starting therapy.

Summary

The optimal way to evaluate subjects with suspected NAFLD continues to evolve. While there is general consensus on several issues, a full consensus is not yet reached on others. These underscore the need for a continuing dialogue between all of the stakeholders in this arena to identify gaps in knowledge and unmet needs and clarify areas of uncertainty in the development of diagnostics and therapies against NASH.

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Pathology of Alcoholic and Nonalcoholic Fatty Liver Disease

12

Pierre Bedossa and David E. Kleiner

Nonalcoholic Fatty Liver Disease

Histopathological Features of NAFLD in Adults

Nonalcoholic fatty liver disease (NAFLD), like ALD, is a heterogeneous collection of injury patterns. These injury patterns share the common feature of steatosis, which is not a specific histological finding on its own. Although in the blinded review of cases in a research setting it may be of value to categorize the various injury patterns into structured and well-defined categories [1, 2], in routine practice, it is sufficient to divide adult NAFLD into two basic categories: NAFLD without features of steatohepatitis and nonalcoholic steatohepatitis (NASH). Because the pathologist often is not informed of the clinical details necessary to separate alcoholic from nonalcoholic fatty liver disease, these two categories become steatosis and steatohepatitis in pathology reports. This distinction is important because steatohepatitis is thought to be the more serious form of NAFLD and is more likely (if left unchanged by effective intervention) to lead to end-stage liver disease [3]. Clinical trials that aim to alter the natural history of NAFLD focus on patients with NASH as they are believed to be at greater risk for disease progression [2].

NASH was originally defined as a disease having the histological appearance of alcoholic hepatitis in a patient who either did not drink alcohol or whose alcohol consumption was minimal [4]. Although the word "steatohepatitis" was coined by the Mayo Clinic group, the pathology had been recognized much earlier in nonalcoholic populations [5, 6]. Currently, patients with fatty liver disease who drink alcohol moderately (less than three drinks per day for men and two drinks per day for women) are included in clinical studies of NAFLD and NASH. The key histological feature distinguishing cases of NASH from steatosis alone is the ballooned hepatocyte [7], discussed further below. In early-stage disease, the ballooning injury is almost always found in acinar zone 3 and is often accompanied by delicate perisinusoidal fibrosis. Steatosis and inflammation are present, but the degree of these findings is variable across cases of NASH. In the subsections below, we discuss the appearance of the various histological features of NAFLD and how these relate to the diagnosis of steatohepatitis.

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Steatosis

Steatosis is the histological feature that ties together all forms of NAFLD. Steatosis is most simply defined as the accumulation of lipid-filled vacuoles within hepatocytes, visible using a light microscope on routinely stained sections without the need for special techniques. Because lipid is removed during the processing of formalin-fixed tissues, the lipid vacuoles have an empty, optically clear appearance when they are larger in diameter than the thickness of the tissue section (about 3–5 μm). Steatosis is a common finding in liver biopsies of patients with liver diseases other than NAFLD. This may be because of risk factors for NAFLD or ALD or because the patient's liver disease is independently associated with steatosis. An example would be the chronic hepatitis associated with genotype 3 hepatitis C [8]. For this reason, steatosis alone is not specific and correlation with clinical information is necessary to know whether the steatosis is related to NAFLD or to some other cause.

Even in patients with no other known liver disease, the amount of steatosis may be so little that it is likely to be irrelevant to any ongoing process. The threshold for significant steatosis has been defined as 5 % of hepatocytes with lipid vacuoles visible at low to medium magnification [2]. It should be recognized that this threshold was based on the expert consensus that some threshold needed to be set to exclude cases with minimal changes from inclusion in patient populations under study for NAFLD. The steatosis in NAFLD is usually macrovesicular, generally with a large dominant vacuole that displaces the nucleus and fills the cell (Fig. 12.1). The lipid accumulation can be so extreme that liver cells may take on an appearance of adipocytes. In early disease, the steatosis often shows a zone 3 distribution, with sparing of periportal hepatocytes [9]. As steatosis accumulates, this zonal distinction disappears and steatotic hepatocytes are present in all of the acinar zones equally.

Although steatosis may be present in a nearly pure form, as the disease progresses to steatohepatitis, other findings, particularly fibrosis, can disrupt the appearance of the steatosis, making the zonal distribution unclear [9]. Steatotic hepatocytes may have a mix of smaller and larger vacuoles and patches of steatosis may be distributed irregularly. As cirrhosis develops, the amount of steatosis decreases and can actually fall below the 5 % threshold, making it difficult to recognize that the disease resulted from NAFLD and NASH. Although many such cases were categorized as cryptogenic cirrhosis in the past, careful examination of the biopsy will usually reveal features of steatohepatitis, particularly ballooned hepatocytes and Mallory–Denk bodies [10, 11]. NASH may recur after transplantation [12].

True microvesicular steatosis, in which hepatocytes take on a foamy appearance with innumerable tiny vacuoles, may be seen sometimes in NAFLD, particularly when steatohepatitis is present (Fig. 12.1). Hepatocytes with this change may be found singly or in small patches. Another steatotic change, in which hepatocytes may show multiple vacuoles of variable size, is a common finding and should not be mistaken for microvesicular steatosis. Microvesicular steatosis tends to be found in more severe cases of steatohepatitis, but its clinical significance as an independent finding is still unclear [13].

Inflammation

The inflammation in NAFLD and NASH is conventionally divided into parenchymal inflammation and portal inflammation. In general, the severity of inflammation, particularly portal inflammation, increases as the disease progresses from steatosis to steatohepatitis, but there is enough variability that the severity of inflammation alone is not helpful in distinguishing steatohepatitis from steatosis alone.

The parenchymal inflammation consists of small foci of lymphocytes and macrophages, often appearing to infiltrate into liver cell plates (Fig. 12.1). These inflammatory foci are very similar to those seen in other chronic liver diseases. When the foci of inflammation are composed mainly of macrophages, they are called microgranulomas. Microgranulomas are a very common finding in NAFLD and sometimes may surround a lipid vacuole, presumably from a hepatocyte that has been destroyed. In this configuration, they resemble the "crown-like

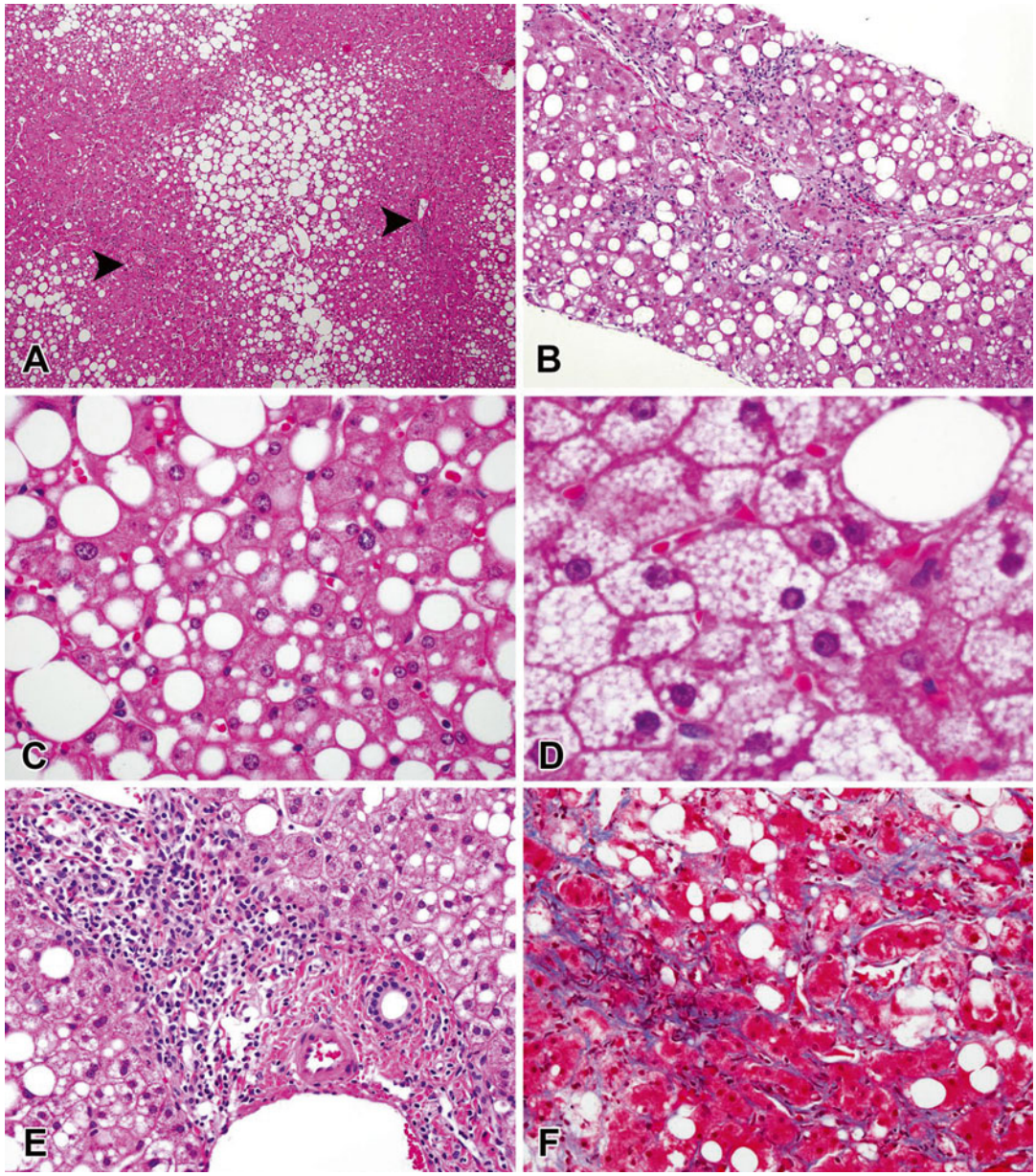

Fig. 12.1 Features of NAFLD. (**a**) Zone 3 distribution of steatosis in a case without fibrosis or features of steatohepatitis. Portal areas are indicated with *arrowheads*. (**b**) Zone 3 injury in a case of steatohepatitis. Steatosis, lobular inflammation, ballooning, and perisinusoidal fibrosis are all present. (**c**) Steatosis in NAFLD is generally macrovesicular, with a mix of small and large vacuoles. (**d**) Patches of microvesicular steatosis may be seen, particularly in severe cases of steatohepatitis. (**e**) Portal inflammation in NAFLD is generally mild or only focally of moderate severity. (**f**) Masson stain showing perisinusoidal fibrosis between the hepatocytes

structures" described in adipose tissue of humans and animals with metabolic syndrome [14]. Apoptotic hepatocytes (acidophil bodies) may be seen associated with parenchymal inflammation or separate from inflammatory foci. They are seen more frequently as the disease severity increases [15, 16]. Foci of neutrophils are not seen as often in NAFLD as in ALD; however,

when present, they are seen close to or surrounding balloon cells and Mallory–Denk bodies.

The portal inflammation in NAFLD and NASH is typically milder than seen in other chronic liver diseases. Most cases will show at least a few portal areas with a sparse infiltrate of lymphocytes and macrophages. As the fibrosis progresses, the number of portal areas with inflammation and the degree of inflammation within individual portal areas increases [17] (Fig. 12.1). This can cause confusion with chronic hepatitis in cases where the steatosis is mild and the balloon cells are not apparent. Plasma cells and eosinophils are rare when present and do not dominate the infiltrate. Interface hepatitis is sometimes seen, particularly when periportal fibrosis is present, but it is mild in comparison to the average case of chronic hepatitis, with focal involvement of a minority of portal areas.

A few studies have evaluated the character of the inflammation in NAFLD using immunohistochemical techniques. Lefkowitch et al. used anti-CD68 to study the distribution of macrophages in cases of NASH [18]. They found that clusters of hypertrophied macrophages seemed to be present more often in zone 3 and tended to be close to ballooned hepatocytes. In NAFLD cases without features of NASH, the aggregates of macrophages were not as prominent. Lymphocytic inflammation in the parenchyma was composed of nearly equal numbers of CD4-(+) and CD8-(+) T lymphocytes with very few B lymphocytes. With respect to the portal inflammation, Gadd et al. noted that the infiltrate was dominated by macrophages and CD8-(+) T cells [19]. Using data from paired biopsies, they suggested that increased numbers of portal macrophages may be present in cases of steatosis that progressed to steatohepatitis.

Ballooning

Ballooning hepatocellular injury is the characteristic cellular lesion of steatohepatitis [7] and current expert consensus suggests that without evidence of ballooning injury, a definite diagnosis of steatohepatitis cannot be made [2]. Because no noninvasive test can accurately detect the presence of balloon cells, a biopsy is required to distinguish NASH from cases with steatosis alone. Balloon cells are typically larger than normal hepatocytes, but they may not be larger than a hepatocyte containing a large fat vacuole (Fig. 12.2). The cytoplasm is irregularly clumped and stranded, leaving irregular translucent or optically clear spaces in between. Small fat vacuoles can sometimes be seen but should not dominate the cytoplasm. Ballooned cells are found most often in acinar zone 3 adjacent to strands of perisinusoidal fibrosis. When the ballooning injury is severe, the balloon cells may be large enough to recognize from low magnification, but it should be recognized that there is wide variation in the size and numbers of balloon cells between individual cases of steatohepatitis. In any case of fatty liver disease with characteristic fibrosis (described below) or clear zone 3-centered injury with steatosis and inflammation, a diligent search should be made for balloon cells. Examination of multiple sections may be necessary in order to find diagnostic cells.

Mallory–Denk bodies have long been recognized as a distinctive feature of ALD and were important in the recognition of NASH as a distinct form of liver disease in nonalcoholics. Mallory–Denk bodies are most easily identified when they appear within ballooned hepatocytes (Fig. 12.2). In such cases, they appear as irregular, densely eosinophilic, ropey cytoplasmic inclusions. Often they are closely associated with the nucleus either as an irregular inclusion to one side or surrounding the nucleus like a collar. Mallory–Denk bodies are composed of a large complex of hyperphosphorylated and misfolded filaments of keratins 8 and 18 [20]. Immunohistochemical staining for ubiquitin, p62, and keratin 8 and 18 can help to identify Mallory–Denk bodies, and these stains will also identify Mallory–Denk bodies in non-ballooned hepatocytes. Recent work has promoted the use of double immunostains for ubiquitin and keratin 8 and 18 to both help identify Mallory–Denk bodies and ballooned hepatocytes [21, 22]. In this stain, the Mallory–Denk bodies are double

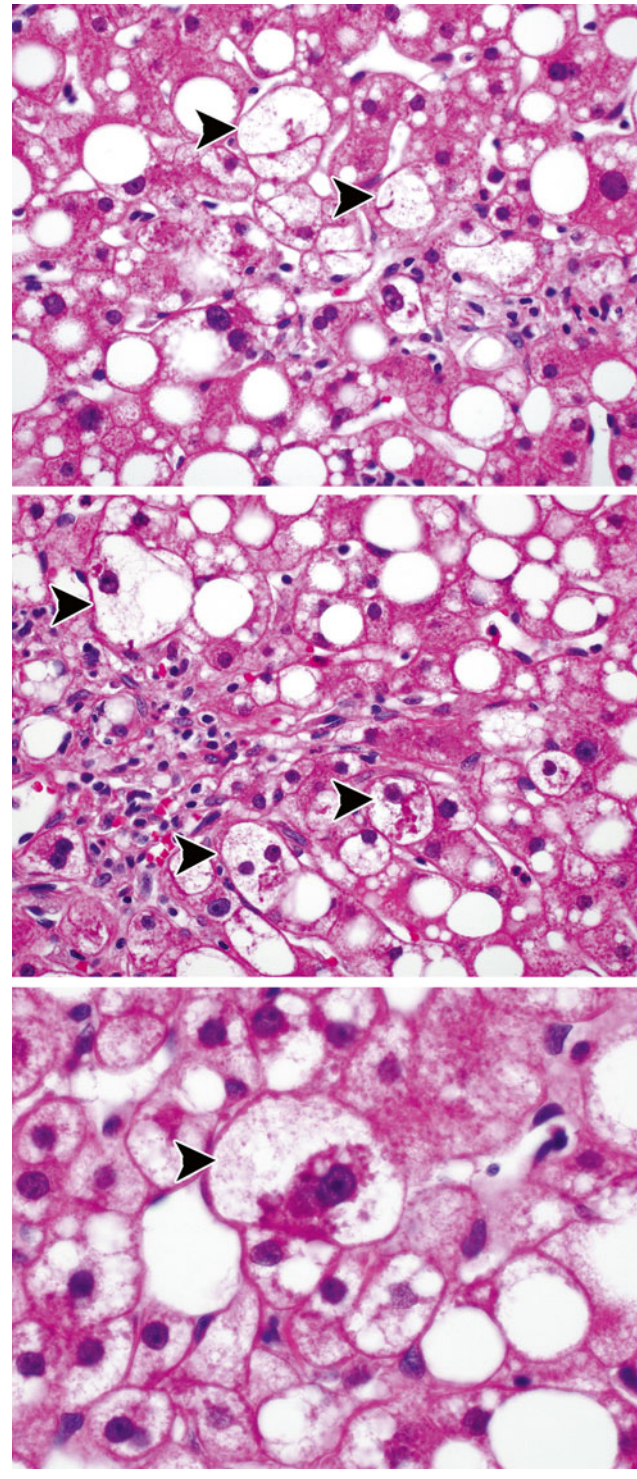

Fig. 12.2 Ballooned hepatocytes. The *panels* show examples of ballooned hepatocytes in NASH (*arrowheads*). Some contain Mallory–Denk bodies

stained and the cytoplasm of ballooned cells will be negative. Since the surrounding normal and steatotic hepatocytes will stain positively with keratin 8 and 18, this double stain can help pathologists to be confident in their identification of balloon cells.

Fibrosis

Like most chronic liver diseases, NAFLD carries a risk to develop fibrosis and cirrhosis. Patients with steatohepatitis, rather than those with just steatosis, are considered to be at risk for cirrhosis and in fact most cases diagnosed as steatohepatitis will have some degree of fibrosis already present. Unlike chronic viral hepatitis and chronic cholestatic diseases, the fibrosis in steatohepatitis begins in zone 3 rather than around portal areas. This fibrosis takes the form of collagen deposition in the perisinusoidal space between endothelial cells and hepatocytes (Fig. 12.1, panel f). This leads to a network of fibrosis that surrounds and isolates individual hepatocytes extending out from the terminal hepatic venule. The earliest stages require the use of special connective tissue stains in order to visualize the fibrosis, but as the fibrotic network thickens and hepatocytes are lost, the fibrosis can be seen on routine stains. Periportal fibrosis develops next in most cases, with periportal hepatocytes trapped by collagen. Ductular reaction may be seen in these cases, particularly if keratin 7 or 19 stains are employed to highlight ductular epithelial cells. Bridging fibrosis follows, with fibrotic connections along zone 3 from portal areas to adjacent terminal hepatic venules. Bridging fibrosis between terminal hepatic venules can be observed, but pure portal–portal bridging fibrosis is unusual in adults with steatohepatitis. Cirrhosis that develops from steatohepatitis can resemble cirrhosis from other forms of chronic liver disease, particularly chronic viral or autoimmune hepatitis. Before NASH was recognized as a significant liver disease leading to cirrhosis, a significant minority of patients presenting to transplant centers were classified as having cryptogenic cirrhosis [11]. Studies of this patient population showed that many of these patients had risk factors for NAFLD and NASH and careful examination of explants revealed residual ballooning and Mallory–Denk bodies even though the degree of steatosis was minimal. Studies of patients with steatohepatitis documented prior to transplant confirmed this observation, suggesting that most patients undergoing transplant for cryptogenic cirrhosis actually had cirrhosis related to NASH [10].

A diagnostic dilemma arises when the biopsy shows steatosis, inflammation, and a characteristic fibrosis pattern but lacks definitive ballooning injury. As noted above, this should prompt a careful search for balloon cells, with the use of immunohistochemical studies for Mallory–Denk bodies if available. However, as with any histological lesion in the liver, the observation of ballooning injury may be subject to sampling adequacy and to observational variability [23]. Since ballooning is a required element for the diagnosis of definite steatohepatitis, one solution is to classify these cases descriptively as fatty liver disease with steatosis, inflammation (if present), and fibrosis but without diagnostic changes of steatohepatitis. Another approach is to classify these cases as having borderline changes of steatohepatitis and there is evidence to suggest that cases classified in this manner have clinical characteristics that fall between those of steatosis alone and definite steatohepatitis [1, 24]. Either approach is valid as long as there is clear communication between the pathologist and clinician and the biopsy is adequately described in the report.

Other Findings

There are a variety of other findings that may be seen in biopsies from patients with NAFLD. These include megamitochondria, cytoplasmic glycogenosis, glycogen nuclei, lipogranulomas, and iron. Of these, glycogenosis requires some attention because it is common and glycogenotic hepatocytes can mimic balloon cells or make them harder to identify. In glycogenosis, the hepatocytes are enlarged and the cytoplasm has a pale, amphiphilic appearance with delicate eosinophilic stranding. Most of the hepatocytes can be affected, resulting in an appearance similar to glycogenic hepatopathy. Glycogenic hepatopathy is an acute form of glycogenosis associated with

type I diabetes and high aminotransferase levels that resolves once glucose levels are controlled [25]. The glycogenosis of NAFLD does not carry the same clinical significance although there is a weak association with type II diabetes [26]. Megamitochondria can be seen in NAFLD as in ALD. They are ovoid eosinophilic, periodic acid–Schiff reaction-negative, cytoplasmic inclusions [13, 27]. Their clinical significance in NAFLD is unclear. Glycogen nuclei are a common finding in NAFLD and NASH and they have been associated with diabetes. They do not have any clinical or diagnostic significance. Lipogranulomas may be seen in NAFLD as in other chronic liver diseases, but they are mainly associated with dietary mineral oil and are seen more frequently in older patients [28]. Because they can be located near terminal hepatic venules and associated with fibrosis, they can cause confusion in early-stage disease. The fibrosis associated with lipogranulomas should be discounted when assessing fibrosis in NAFLD.

Iron is often an incidental finding in biopsies performed to evaluate NAFLD. Because iron may act as a cofactor in hepatic disease progression, Nelson et al. examined the relationship of iron deposition and histological severity in NAFLD in a large patient cohort [29]. They found an association between iron accumulation in Kupffer cells and the severity of inflammation, ballooning, and fibrosis. Biopsies showing only hepatocellular iron had less injury than those with no stainable iron. The pathophysiology behind these differences is still unclear, although the authors speculated that there may be differences in hepcidin signaling that could lead to iron deposition in different cells.

Histopathological Features of NAFLD in Children

Children, particularly adolescents, may develop the same histological spectrum of NAFLD as adults, although the incidence of cirrhosis is less and the disease tends to be milder overall. Preadolescent children also have a form of fibrosing fatty liver disease with a different histological appearance than NAFLD in adults and adolescents [30]. This type of NAFLD was originally called type 2 steatohepatitis, but the NASH Clinical Research Network uses the term "zone 1 borderline pattern" because most cases lack typical balloon cells and so do not meet criteria for steatohepatitis [1]. This latter term also highlights the characteristic histological change—zone 1 predominant injury. Cases show the most severe macrovesicular steatosis in periportal hepatocytes, with decreasing amounts of fat as the terminal hepatic venules are approached (Fig. 12.3). The steatosis may also be pan-acinar, but the terminal hepatic venule region will be spared in terms of inflammation and fibrosis. The portal areas contain a mild, lymphocytic, and histiocytic infiltrate that is often associated with periportal fibrosis. Bridging fibrosis involves adjacent portal areas and the terminal hepatic venules are uninvolved. Connective tissue stains can be very helpful in bringing out the zone 1 injury pattern by highlighting early septum formation. Balloon cells are rarely found and are not as well formed as those seen in adult-type steatohepatitis. Mallory–Denk bodies are not seen, and their presence should suggest the diagnosis of steatohepatitis rather than the zone 1 pattern. This pattern is common among children with fatty liver disease, comprising 28 % of a cohort of US children between age 6 and 17 [31]. Among adults, blinded review finds only 1 % of cases to show the zone 1 injury pattern [24, 32]. Patton et al. examined the clinical characteristics of children with the borderline zone 1 pattern in comparison to children with definite steatohepatitis [31]. The zone 1 group were younger (mean age 11 vs. 13), less physically mature (Tanner stage 1.8 vs. 2.7), and more often of Hispanic ethnicity (69 % vs. 46 %) and had lower fasting insulin levels (23 vs. 36 mg/dL) with the same fasting glucose levels. The natural history of this form of fatty liver disease is unclear. The fact that the zone 1 pattern is not seen as often in adolescents suggests that either the disease shifts to zone 3 or resolves without progression.

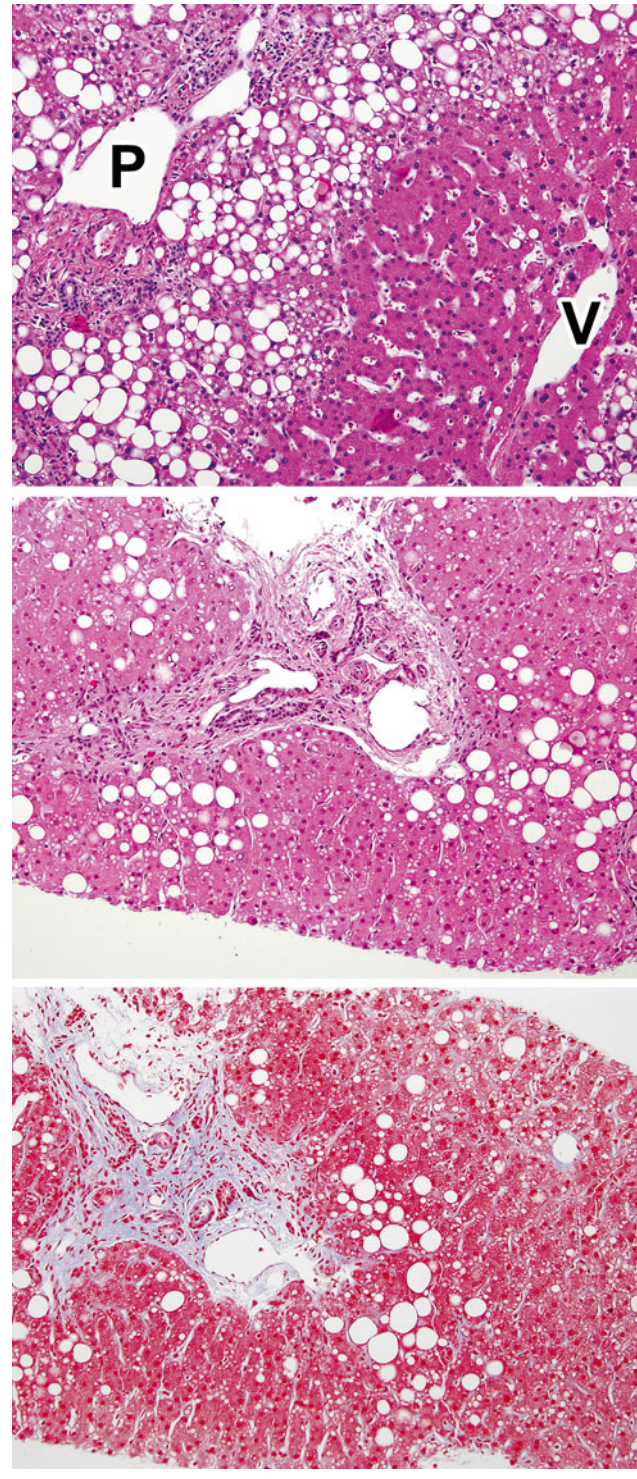

Fig. 12.3 Zone 1 borderline pattern. These *panels* illustrate the zone 1 pattern of injury seen most often in young children. In the *top panel*, there is moderate steatosis that hugs the edge of the portal area (P), while the terminal hepatic venule (V) shows no steatosis or injury. The *bottom panels* show H&E and Masson trichrome stained parallel sections with mild zone 1 steatosis associated with periportal fibrosis

NAFLD in Patients with Lipodystrophy

Lipodystrophies are a collection of disordered characterized by loss of adipose tissue (particularly from the subcutaneous compartment). The loss may be partial or generalized and both genetic and acquired forms are described [33]. The acquired forms may be related to drug injury, particularly with certain antiretroviral medications [34], or idiopathic, with a presumed autoimmune etiology. Patients have insulin resistance that is frequently severe, low adiponectin and leptin levels, and severe hypertriglyceridemia with low levels of high density lipoproteins. Most have NASH, probably related to their insulin resistance [35]. A recently published study described the liver biopsy findings in 50 patients (including both adults and children) with various forms of lipodystrophy [36]. Within this group, 82 % had definite or borderline steatohepatitis and 42 % had advanced fibrosis (bridging fibrosis or worse). The most advanced cases (and all cases of cirrhosis) were seen in patients with acquired generalized lipodystrophy and in patients with congenital generalized lipodystrophy who had a mutation in the seipin gene. In other respects, the character of the NAFLD in lipodystrophy was similar to that seen in patients with more common risk factors. Although fatty liver disease was the most common finding, four patients had histological and clinical evidence of autoimmune hepatitis. The cohort was treated with metreleptin in an open-label study since leptin therapy is known to ameliorate the insulin resistance in patients with lipodystrophy [37]. Follow-up biopsies performed in 27 patients showed reduction in steatosis, lobular inflammation, ballooning, and some resolution of steatohepatitis. Fibrosis remained unchanged in this population.

Steatosis and Steatohepatitis in Patients with Other Chronic Liver Diseases

Because steatosis is a common finding in liver biopsies, it is not unusual to find steatosis and more specific features of steatohepatitis in patients with other liver diseases. Steatosis and steatohepatitis may have diverse etiologies when found as a second disease process. Risk factors for ALD and NAFLD may be present, either alone or together. The disease process itself may increase the likelihood of steatosis for other reasons. The well-known association of infection with genotype 3 of hepatitis C and steatosis is probably the best example [8, 38]. Patients may be on medications such as methotrexate or tamoxifen that are associated with fatty liver disease [39]. Drugs may also cause weight gain [34, 40, 41] or lipodystrophy [42, 43], leading to steatosis by a secondary mechanism.

In one large single-center study, steatohepatitis was found as a complication in 5.5 % of cases of chronic hepatitis C and 4 % of other liver biopsies performed for other liver diseases [44]. Among the hepatitis C cases, 27 % had alcohol as a risk factor, but 60 % of these also had obesity and 25 % had diabetes as concurrent risk factors. Only 7 % were infected with genotype 3. Criteria for diagnosing concurrent steatohepatitis in the presence of other liver diseases should be strict, with unequivocal ballooning injury (Fig. 12.4). It is very helpful to find Mallory–Denk bodies and perisinusoidal fibrosis along with the balloon cells. Identification of concurrent steatohepatitis should prompt a search for the etiological risk factors and should be taken into account when using biochemical serum measurements to assess response to therapy of the primary disease.

Although excessive alcohol consumption is consistently identified as a risk factor for disease progression in chronic liver disease from other causes, evidence for a similar effect of NAFLD on disease progression has been more difficult to define. Most of the published studies have focused on chronic hepatitis C and have been limited to examining the relationship of steatosis, rather than steatohepatitis, on fibrosis progression. In cross-sectional studies, steatosis (mainly related to metabolic syndrome) has been associated with the stage of fibrosis [45–47]. The I148M polymorphism of the patatin-like phospholipase domain-containing 3 (PNPLA3) gene, which has been highly linked to NAFLD and NASH, has also been linked to increased cirrhosis prevalence in cross-sectional studies of hepatitis C [48]. A longitudinal study of

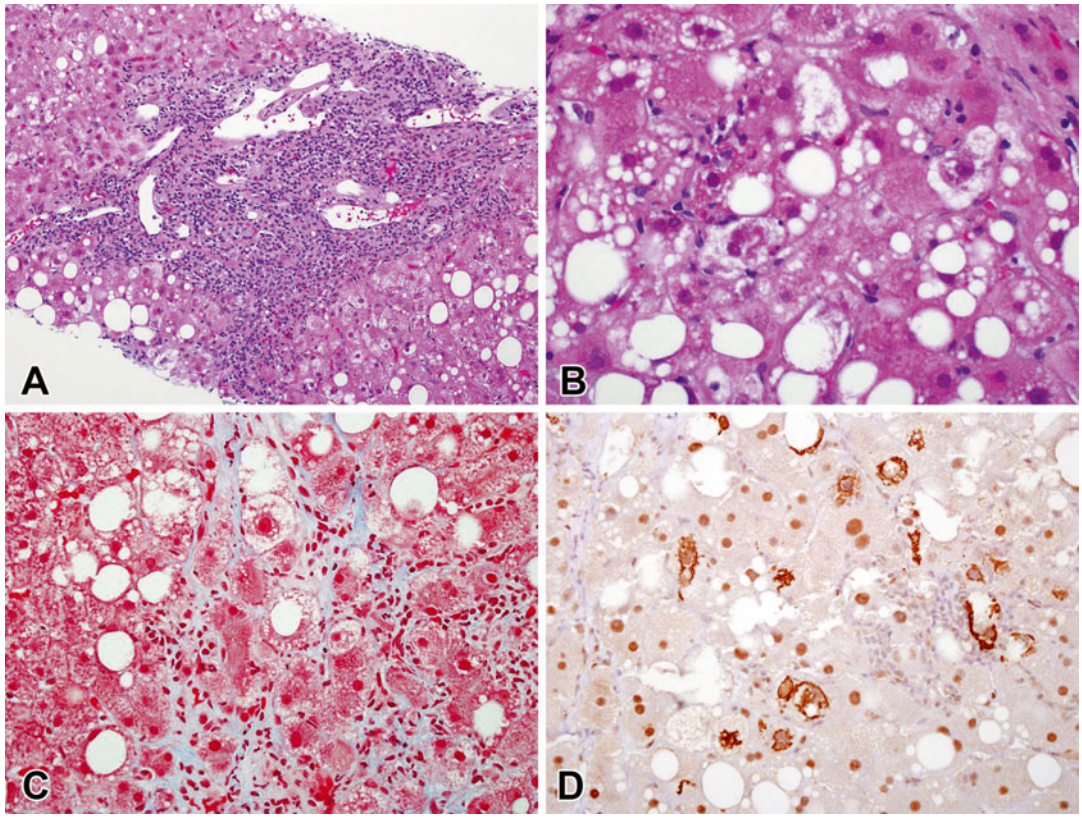

Fig. 12.4 Steatohepatitis in a case of chronic hepatitis C. (**a**) There is dense portal inflammation with interface hepatitis consistent with hepatitis C infection. Steatosis is present. (**b**) In zone 3, ballooning injury with Mallory–Denk bodies is seen. (**c**) The Masson trichrome stain showed characteristic perisinusoidal fibrosis in zone 3. (**d**) A ubiquitin immunostain highlights the many Mallory–Denk bodies that were present

chronic hepatitis C using paired biopsies failed to find an association between steatosis and progression of fibrosis despite confirmation of an association in cross-sectional analysis [49]. An association between fibrosis and steatosis has been reported in primary biliary cirrhosis [50] but not chronic hepatitis B [51, 52]. The potential relationship between NASH and fibrosis in other chronic liver diseases has not been studied extensively, possibly because of the much lower prevalence of NASH than steatosis.

Natural History of NAFLD in Biopsy Studies

Biopsy remains the reference tool for assessing the histopathological patterns and natural history of NAFLD since noninvasive markers have neither been fully validated in this context nor do they provide information on the whole histologic spectrum of NAFLD. However, as for any chronic liver disease, the liver biopsy has inherent limitations. Among them, sampling error is a significant concern. Indeed, the liver biopsy samples only a very tiny part of the whole organ and there is a risk that this part might not be representative of fibrosis and other lesions due to heterogeneity in disease distribution. In the context of NAFLD and in morbid obesity, several studies have underlined this risk although the magnitude of discrepancies is variable according to histological features and across the different studies [23, 53–56]. As in other chronic liver diseases, the risk of sampling error is decreased with increasing length of the liver biopsy. Except in the diagnosis of cirrhosis, for which micro-fragments may be sufficient, a 25 mm long biopsy is

considered an optimal specimen for accurate evaluation, though 15 mm is considered sufficient in most studies [57]. The caliber of the biopsy needle is also important for getting an adequate biopsy of liver for evaluation. A 16 gauge needle is considered to be appropriate [58]. Another potential limitation is related to the discordance between pathologists in biopsy interpretation (interobserver variation), although it is less important when biopsy assessment is done by specialized liver pathologists [59].

The natural history of NAFLD is an active area of investigation. The broad outlines are clear—that NAFLD and particularly NASH are chronic liver diseases that may progress to cirrhosis. The details remain to be fully characterized. What patients are at greatest risk for fibrosis progression over the short and over the long term? What determines whether a patient with steatosis alone develops NASH or fibrosis? Does disease activity (and/or fibrosis) fluctuate with time, and if so, what clinical factors drive the improvement or worsening of disease? Understanding the short-term behavior of this disease has bearing on deciding which patients are most likely to benefit from a potential therapy, since most clinical trials test efficacy over a year or two. Given the metabolic nature of NAFLD, the treatment paradigm will ultimately be more like that of hypertension and diabetes than an infection like chronic hepatitis C.

Although some of the long-term issues can be examined by retrospective cohort studies with hard end points (death, transplantation, cancer), information useful for patient management is better derived from longitudinal studies where pairs of biopsies can be examined. A number of paired biopsy studies have been published in NAFLD, almost all of them in adult populations. These studies fall into two groups. One group consists of cohorts of patients collected retrospectively or prospectively and the outcome examined is fibrosis progression. The other group consists of placebo-treated patients in clinical trials. In this latter group, the outcome is usually fibrosis regression or other improvements in histological findings. Longitudinal cohorts typically have longer time intervals between the biopsies, on the order of 3–6 years, while placebo groups have a time between biopsies limited by the treatment period.

In one of the larger cohort studies published to date, Sorrentino et al. looked at paired biopsies in 132 patients [60]. With a mean time between biopsies of 6.4 years, they observed fibrosis progression in 45 and regression in 11. In their study, which was focused on the evaluation of fibronectin staining in baseline biopsies, they found that the degree of fibronectin staining, the presence of hypertension, and a HOMA-IR score of >10 were associated with increased risk of fibrosis progression. Although early cohort studies suggested that patients with steatosis alone or steatosis with mild inflammation were at little or no risk to progress, Pais et al. recently reported data on a cohort of 25 patients with NAFLD (but not NASH), 16 of whom developed steatohepatitis over a mean follow-up of 3.7 years [61]. Six of these patients developed bridging fibrosis. These data suggest that assumptions about benign clinical course cannot be made in patients who only have steatosis and inflammation on biopsy although differences in progression rates are likely to exist between patients with steatohepatitis and those with only steatosis. The NASH Clinical Research Network also recently presented data on their longitudinal cohort of 375 adults with paired biopsies followed for a median time of 4 years. In this group, 128 (36 %) showed fibrosis progression and 106 (28 %) showed fibrosis regression [62]. Although baseline clinical data was of no value in predicting which patients would progress or regress, changes in BMI, aminotransferases, and alkaline phosphatase were all clearly associated with changes in fibrosis.

The PIVENS clinical trial of vitamin E and pioglitazone is one of the largest placebo-controlled studies to publish information on its placebo group [63]. Out of 72 placebo-treated patients, 22 had apparent regression of fibrosis. While some "improvement" in fibrosis can be attributed to observational variability, these patients also improved clinically, with loss of weight in some and decreased aminotransferases overall. Others [64–66] have also reported improved histological findings in placebo groups. These findings, along with the findings of fibrosis

progression and regression in longitudinal cohorts, suggest that NAFLD may have a fluctuating natural history depending on patient-initiated lifestyle modifications and other factors that may be difficult to model or anticipate.

Staging and Grading in NAFLD

Because the liver biopsy remains important in the evaluation of the natural history and in clinical trial outcomes, it is important to have structured methods to assess histological change in the liver biopsy. Like chronic hepatitis, there are several staging and grading systems available for use in clinical research. These systems can also guide the evaluation of the liver biopsy in daily practice, although there should be clear communication between the pathologist and the clinical staff about which system is being reported.

The first system published was that of Brunt et al., who proposed a system for grading and staging NASH (Table 12.1) [67]. A prerequisite for applying this system was a diagnosis of steatohepatitis, after which the degree of steatosis, portal and lobular inflammation, and ballooning could be combined to provide a final grade of mild, moderate, or marked activity. Fibrosis in this system was staged from 0 to 4 as shown in Table 12.1. Several years later, when the NASH Clinical Research Network was formed, the pathologists in the network were tasked with creating a scoring system

Table 12.1 Central elements of three grading and staging systems for NAFLD and NASH

Feature	Grade/ score	Grading/staging system		
		Brunt[a] [67]	NASH-CRN [17, 68]	SAF [72]
Steatosis	0	None	<5 %	<5 %
	1	≤33 %	5–33 %	5–33 %
	2	33–66 %	33–67 %	33–67 %
	3	>66 %	>67 %	>67 %
Lobular inflammation	0	No foci	No foci	No foci
	1	1–2 foci per ×20 field	<2 foci per ×20 field	<2 foci per ×20 field
	2	2–4 foci per ×20 field	2–4 foci per ×20 field	>2 foci per ×20 field
	3	>4 foci per ×20 field	>4 foci per ×20 field	
Ballooning	0	None	None	Only normal hepatocytes
	1	Mild, zone 3	Few	Few: clusters of hepatocytes with rounded shape and reticulated cytoplasm
	2	Prominent, zone 3	Many	Many: enlarged hepatocytes (≥2× normal)
	3	Marked, zone 3		
Portal inflammation	0	None	None	
	1	Mild	Mild	
	2	Moderate	More than mild	
	3	Severe		
Fibrosis (stage)	0	None	None	None
	1	Zone 3 perisinusoidal fibrosis only	Perisinusoidal or periportal fibrosis; 3 substages defined	Perisinusoidal or periportal fibrosis
	2	Zone 3 perisinusoidal fibrosis and periportal fibrosis	Perisinusoidal and periportal fibrosis	Perisinusoidal and periportal fibrosis
	3	Bridging fibrosis	Bridging fibrosis	Bridging fibrosis
	4	Cirrhosis	Cirrhosis	Cirrhosis

[a]The Brunt system is meant to be applied only after a diagnosis of steatohepatitis is made

that could be used in the natural history studies and clinical trials of the network [68]. The system was an intellectual successor to the Brunt system but extended it so that it could be applicable to any patient, adult or child, that the network might enroll. Selected parts of the system are shown in Table 12.1. An aggregate score, the NAFLD Activity Score (NAS), was defined as the unweighted sum of the steatosis, lobular inflammation, and ballooning scores based on the fact that those features were most closely associated with the diagnosis of NASH. Fibrosis was not included in this score so that the activity score could be distinct from the stage. The aggregate score has proved useful in clinical trials and other clinical studies to demonstrate histological improvement and to correlate with other findings [24, 63, 69, 70]. Patients in the PIVENS clinical trial who showed improvement in NAS also showed improvement in fibrosis [71].

The Fatty Liver Inhibition of Progression (FLIP) consortium has adopted a new system for use in its studies [59]. This system, first published by Bedossa et al., also assesses steatosis, lobular inflammation, ballooning, and fibrosis (Table 12.1) [72]. The scores can be abbreviated for reporting purposes in "SAF" form, where each letter is followed by a number indicating steatosis (S) grade, activity (A), and fibrosis (F) stage. Activity is defined as the sum of the lobular inflammation and ballooning scores. An algorithm, shown in Table 12.2, relates histological findings and scores to diagnosis. Because the SAF system simplifies the relationship between scores and diagnosis, the reproducibility of diagnostic categorization is enhanced [72]. Although other systems have been published [73, 74], they have not garnered the attention of the ones noted above. Because the existing systems (even those not described in detail here) are similar, the selection of one system or another should depend mainly on the familiarity of the pathologist with the system and on the purpose for which it is to be used. All of these systems are semiquantitative in nature and could be supplemented in clinical research with quantitative image analysis, particularly for quantification of steatosis and fibrosis.

Table 12.2 The SAF diagnostic algorithm for NAFLD and NASH [72]

Steatosis	Ballooning	Lobular inflammation	Diagnosis
0	0, 1 ,or 2	0, 1, or 2	Not NAFLD
1, 2, or 3	0	0	NAFL
		1	NAFL
		2	NAFL
	1	0	NAFL
		1	NASH
		2	NASH
	2	0	NAFL
		1	NASH
		2	NASH

Alcoholic Liver Disease

Introduction

Alcoholic liver disease (ALD) is the third most common risk factor for disease and disability worldwide (World Health Organization, Global Health Observatory. Prevalence of alcohol use disorders. United States: World Health Organization, 2004). Almost 4 % of all deaths in the world result from ALD [75]. Within the spectrum of chronic liver diseases, ALD constitutes the leading cause of liver cirrhosis, the most common cause of hepatocellular carcinoma (HCC) in the Western countries [76] and the second most common indication for liver transplantation [77].

The histological spectrum of ALD is multifaceted but can be summarized in three main schematic patterns: steatosis, alcoholic hepatitis, and fibrosis/cirrhosis. These patterns are not distinct entities but rather a spectrum of overlapping injuries that can be simultaneously present in different combinations. Most of the lesions are shared with NAFLD but may vary with respect to background physiopathology, severity, and prognosis. Indeed, it is accepted that the overall histopathological appearance is usually milder in NASH than in ASH. However, since the histological changes are similar in both diseases, clinical correlation is of utmost importance in helping

Table 12.3 Similarities and differences in histological features of NAFLD and ALD

Present both in ALD and NAFLD	Evocative of ALD	Evocative of NAFLD
Zone 3 predominance	Sclerosing hyaline necrosis	Glycogenated nuclei
Lobular inflammation	Extensive neutrophilic infiltration	Predominant lobular mononuclear cell infiltrate
Macrovesicular steatosis (may be absent in advanced stages)	Alcoholic foamy degeneration	Ductular proliferation
Portal inflammation (optional)	Venous phlebitis (portal or central vein)	Predominance of periportal lesions in a subset of patients
Hepatocellular ballooning	Phlebosclerosis of central vein	
Mallory–Denk bodies	Cholestasis (canalicular or ductular)	
Apoptotic bodies	Pericholangitis	
Perisinusoidal fibrosis	Severe steatohepatitis with abundant Mallory–Denk bodies	

to define the exact etiology. Finally, there are some additional features that have been described in ALD but not in NAFLD so far and which will be also emphasized in this chapter. Table 12.3 underscores the main pathologic features that may help to orient the diagnosis.

The diagnosis of ALD relies on evidence of liver disease in combination with significant alcohol intake and in absence of other comorbidities. Although liver biopsy remains the standard for assessing the type and extent of liver damage, there is a lack of consensus about performing liver biopsy in patients suspected of ALD, given concerns regarding the risk of sampling error and the related safety of liver biopsy [78]. The recent EASL guidelines state that liver biopsy should not be performed in all patients with ALD but is indicated to confirm the diagnosis and to assess the severity of the disease in cases of aggressive forms of ALD requiring intervention and in situations where cofactors may be contributing to the onset of liver disease [79]. Moreover, liver biopsy is useful in determining the outcome of patients affected by ALD, given that a histological diagnosis of steatohepatitis or cirrhosis is associated with an increase in mortality of at least 50 %, in comparison with simple alcoholic steatosis [80].

Steatosis

Steatosis is the earliest and most common manifestation of alcoholic liver disease and is seen in up to 90 % of ALD patients [81]. The accumulation of lipid droplets containing primarily triglycerides, the natural end product of fatty acid metabolism, but also free fatty acids and other components within the hepatocellular cytoplasm is generally asymptomatic or may be associated by mild disturbance of liver function tests. This lesion is considered quickly reversible with alcohol abstinence. Although a minimum of 5 % hepatocytes containing lipid vacuoles is required for the diagnosis of steatosis in NAFL, this is not necessary in the context of ALD. Steatosis occurs predominantly in hepatocytes adjacent to the terminal hepatic venule (acinar zone 3). As the liver injury progresses, steatosis can be seen diffusely throughout the lobule. When steatosis is massive, the liver is often enlarged and smooth in addition to having a characteristic yellow appearance on gross examination.

Macrovesicular steatosis is the most common form of steatosis in ALD. Lipid droplets exceed 20 μm in diameter and displace the hepatocyte nucleus at the cell periphery. Droplets of smaller size (mediovesicular steatosis) are also frequently associated in a mixed pattern. It is usually considered that fat initially accumulates in the form of small vacuoles, and with time and continued accretion, these progressively coalesce into larger globules.

Rupture of hepatocytes containing fat may result in formation of lipogranulomas. Lipogranulomas consist of an extracellular lipid drop encircled by a loose aggregate of macrophages and histiocytes with occasional lymphocytes, eosinophils, plasma cells, or multinucleated giant cells [82]. Lipogranulomas occur mainly in liver

lobules predominantly in the region of the terminal hepatic venules but can also be present in portal tracts [83]. Rarely, true epithelioid granulomas are seen. Lipogranulomas may be associated with small foci of fibrous scarring, but these are not thought to be important in the progression of fibrosis [82].

Giant mitochondria (megamitochondria) may be seen on light microscopy as eosinophilic, globular, cytoplasmic inclusions (Fig. 12.5). Needle-shaped forms of giant mitochondria are also recognized. Megamitochondria are considered to result from increased mitochondrial membrane permeability in the context of marked oxidative stress. They are identified with a prevalence that ranges in different series from 25 to 90 % in ALD [84, 85]. Giant mitochondria have been suggested as an adverse prognostic feature in cases that otherwise have simple steatosis. Although giant mitochondria are most typically seen in ALD, they have also been described in NAFLD as mentioned previously but they tend to be fewer in number and lack a zonal distribution. Therefore they have been considered as a diagnostic hint of chronic alcohol consumption.

Steatosis per se is not a strong stimulus for fibrogenesis but it is a lesion that needs follow-up since some studies suggest a more rapid progression to fibrosis in ALD [86]. Indeed, 20 % of patients who have developed steatosis and do not cease alcohol intake develop fibrosis and cirrhosis [87]. Patients with a mixed droplet pattern of steatosis have also been found to have a higher risk of progressing to advanced liver disease than those with pure macrovesicular steatosis [86].

Alcoholic foamy degeneration is a feature reported, so far, only in ALD [88]. In this pattern, hepatocytes have a foamy appearance due to accumulation of tiny fat droplets in hepatocyte cytoplasm of acinar zone 3 occasionally spreading to zone 2 in the absence of alcoholic hepatitis or any inflammation. Canalicular bilirubinostasis is frequently seen in perivenular hepatocytes and may mimic extrahepatic biliary obstruction. Patients typically present with jaundice and hepatomegaly. Rapid recovery occurs on abstention from alcohol [89].

There are a few enigmatic cases where sudden death was related to severe steatosis [90]. Postmortem histology showed a predominantly microvesicular or mixed pattern of steatosis without typical features of steatohepatitis [91].

Acute Alcoholic Hepatitis

Steatosis can also evolve with the development of inflammation and hepatocellular injury to alcoholic steatohepatitis (ASH). It encompasses a wide spectrum of clinical and pathologic features, ranging from a mild or unapparent condition to a severe, progressive, and potentially life-threatening disorder with cirrhosis as the end-stage consequence. There is evidence that the natural history of ASH appears to be much more "aggressive" than that of NASH. Acute alcoholic hepatitis (AAH) refers to a severe type of alcoholic steatohepatitis although for some authors, both terms are interchangeable. The clinical syndrome is characterized typically by a history of excessive alcohol consumption and a recent onset of deep jaundice, abdominal pain, fever, and increased white blood cell count which can lead to progressive liver failure and impaired blood clotting. Although clinical manifestations in the typical form may be suggestive, histology remains the gold standard in diagnosing AAH since it may be asymptomatic in some patients. AAH is associated with a high short-term mortality of 35 % if left untreated [92]. In patients with severe AAH, the risks of performing a percutaneous liver biopsy are increased because of coagulation abnormalities and a transjugular route may be required [93]. This procedure is safe and efficient since sufficient material allowed a histological diagnosis to be made in 96 % of samples [94].

Histologically, AAH was originally defined by an international consensus group as the association of steatosis, hepatocyte injury (typically hepatocyte ballooning and apoptosis), and polymorphonuclear infiltration [95]. Mallory–Denk bodies and intraparenchymal cholestasis are often observed but not necessary for diagnosis [96]. These basic changes vary greatly in their severity, extent, and relative proportion (Fig. 12.5).

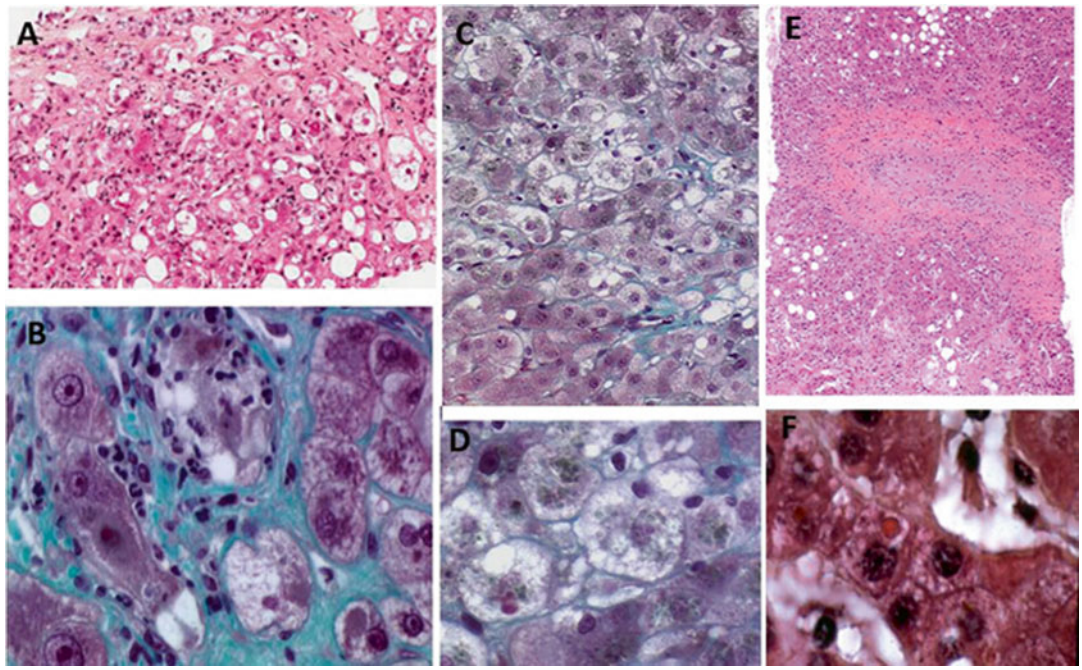

Fig. 12.5 Histological patterns of alcoholic liver diseases. (**a**) Acute alcoholic hepatitis with a diffuse polymorphonuclear cell infiltrate, several Mallory–Denk bodies, and hepatocellular ballooning. (**b**) Satellitosis; polymorphonuclear cells are gathered around clarified hepatocyte with remnant of Mallory–Denk body. (**c**) Alcoholic foamy degeneration. Diffuse clarification of hepatocyte without macrovesicular steatosis. (**d**) Alcoholic foamy degeneration. Higher magnification shows spongiocytic hepatocytes with small intracellular bile plugs. (**e**) Obliterating thrombophlebitis in acute alcoholic hepatitis. The central vein is totally occluded. (**f**) Megamitochondria: dense round highly eosinophilic spot in hepatocyte cytoplasm

The background liver may also display the various changes of ALD from simple steatosis to cirrhosis. In AAH, steatosis is usually present but is variable in severity and may be even absent in patients who have been abstinent prior to the biopsy or in severe form of AAH. Therefore steatosis is not a diagnostic requirement, unlike in NASH.

The hepatocyte injury is characterized primarily by ballooning degeneration. Focal hepatocyte necrosis and acidophilic bodies are also noted, but usually in sparse numbers. As described in NAFLD, ballooned hepatocytes are typically rounded rather than polygonal in shape. The cytoplasm undergoes clarification. In contrast with hepatocytes with steatosis, where a single large fat droplet completely displaces the normal cytoplasm, ballooned hepatocytes have a reticulated appearance made of delicate strands of residual cytoplasmic material with nuclei usually remaining in central position. In some cases, clumps of cytoplasmic material form larger rope-like aggregates characteristic of Mallory–Denk bodies. They are often found in hepatocytes that are much larger than normal hepatocytes. Although a hallmark of alcoholic hepatitis, Mallory–Denk bodies are neither an invariable nor specific feature. They are noted in 40 % to over 80 % of cases in various series, tending to be more prevalent in cases of greater severity. Mallory–Denk bodies persist in liver cells for many months after alcohol consumption ceases. Apoptotic hepatocytes may be noted but are rarely prominent in this context as it is for focal confluent necrosis that might be occasionally seen in acinar zone 3.

In alcoholic hepatitis, the inflammatory infiltrate is typically made of neutrophils that gather around injured hepatocytes. A typical feature is the so-called satellitosis in which neutrophils encircle massively swollen hepatocytes containing

Mallory–Denk bodies [97] (Fig. 12.5). Lymphocytes and macrophages may also enter portal tracts and spill into the parenchyma. A predominantly portal lymphocytic infiltrate can occur in ALD in the absence of any viral or autoimmune marker. However, although minor degrees of portal inflammation and interface hepatitis may be attributed to alcohol, when the portal inflammation is severe, associated comorbidities should be excluded. In addition, Kupffer cells engulf hepatocellular debris arising from hepatocellular death. Thus, the parenchyma in alcoholic hepatitis includes a mixed inflammatory infiltrate with increased numbers of large macrophages. Mallory's hyaline and megamitochondria are suggestive of active drinking [98]. Usually, the inflammation and hepatocellular damage of alcoholic hepatitis is much more severe than the occasional ballooned hepatocytes and the rare Mallory bodies and apoptotic hepatocytes observed in NAFLD although features of varying severity may be encountered. Alcoholic hepatitis is almost always accompanied by prominent activation of stellate cells between altered parenchymal cells and portal tract fibroblasts giving rise to pericellular and portal fibrosis.

The severity of AAH is highly variable. Mild forms are characterized by only occasional foci of liver cell necrosis in the acinar zone 3 regions accompanied by a slight neutrophil polymorph infiltrate, occasional enlarged hepatocytes that may contain incompletely formed Mallory–Denk bodies, and minimal pericellular fibrosis. In fully developed AAH, hepatocyte necrosis is more widespread and sometimes confluent. Hepatocyte enlargement is a prominent feature and Mallory–Denk bodies are numerous. The neutrophil polymorph infiltrate is often concentrated around hepatocytes containing Mallory–Denk bodies but can also be seen within the sinusoids and in the portal tract inflammatory infiltrate. In severe AAH, periportal ductular reaction and cholestasis are often seen. This feature is not reported in NASH and cholestasis due to sepsis should be ruled out on clinical grounds.

As with any histological diagnosis, there still remains interobserver error in the assessment of AAH. In patients with severe AAH and background cirrhosis, this error has been shown to be minimal in one study with a high degree of concordance between two histopathologists ($\kappa = 0.77$) [99].

Patients with AAH not only have a short-term deleterious prognosis but also are at high risk to develop cirrhosis [100]. Indeed, among the ALD-associated histological features, AAH has the highest risk of fibrosis progression leading to cirrhosis in 40 % of cases [101]. In one study, 50 % of patients with alcoholic hepatitis who continued drinking developed cirrhosis in 10–13 years [102]. Conversely, studies which included histological diagnosis as an entry requirement have shown a variation in the prevalence of cirrhosis in patients with AAH from 65 to 95 % [103, 104]. In patients with alcohol-related cirrhosis, AAH can be the precipitating cause of acute-on-chronic liver failure (ACLF) [105–107].

There is evidence that some of the histological patterns as well as soluble circulating factors can predict clinical outcome. This could assist clinical decision making and guide treatment choices. Among histological features, steatosis <20 %, particularly if it is microvesicular, is an independent predictor of poor outcome [108]. Polymorphonuclear cell infiltrate is associated with severity of AAH [109]. The severity of hepatocyte necrosis and extent of pericellular and perivenular fibrosis and the formation of fibrous septa with elastic fiber deposition and architectural distortion are also deleterious prognostic factors [110, 111]. If sepsis is excluded, cholestasis is associated with a worse clinical outcome and is an independent predictor of 3-month mortality [112]. Interestingly, a recent publication reported histological features associated with disease severity and proposed a histologic scoring system to predict short-term (90-day) mortality. The system was tested in a test set of 96 patients from 5 academic centers, and a semiquantitative scoring system called the Alcoholic Hepatitis Histologic Score (AHHS) was developed. The system was validated in an independent set of 109 patients. In the multivariate analysis, stage of fibrosis, PMN infiltration, type of bilirubinostasis, and presence of megamitochondria independently predicted 90-day survival [113]. Further

studies in this area are required to establish a reliable and reproducible scoring system mixing clinical, biological, and histological data that predicts clinical outcome.

Sclerosing hyaline necrosis was described by Edmondson et al. [114]. This feature has not been described so far in NAFLD and is considered as part of the spectrum of severe AAH. It is characterized by dense perivenular, partly obstructive fibrosis associated with Mallory–Denk bodies and residual apoptotic liver cells in acinar zone 3. It results in a large perivenular scar that may occlude the terminal hepatic venules. Portal hypertension may develop in the absence of cirrhosis or even bridging fibrosis.

Three types of venous lesions, phlebosclerosis, lymphocytic phlebitis, and veno-occlusive disease, have been described in a review of 200 autopsy cases of alcoholic liver disease [115]. Lymphocytic phlebothrombosis was noted in 16 % of patients with alcoholic hepatitis and in 4 % of those with cirrhosis (Fig. 12.5). Phlebosclerosis was found in all cases of alcoholic hepatitis and cirrhosis. It may result from advanced perivenular thickening with varying degrees of narrowing of the hepatic vein lumen (Fig. 12.5) [116]. Veno-occlusive lesions were found in 52 % of cases of AAH with portal hypertension. Portal hypertension correlated significantly with the degree of phlebosclerosis and veno-occlusive change. These occlusive venous lesions may contribute to the atrophy of hepatic parenchyma and functional impairment in advanced ALD. These vascular lesions have not been described so far in NAFLD.

Fibrosis and Cirrhosis

Perivenular fibrosis is thought to be the first lesion in a sequence of events which leads ultimately to the development of cirrhosis [117]. It has been defined as fibrosis extending around at least two-thirds of the perimeter of the terminal hepatic venule and measuring at least 4 μm in thickness. This lesion was considered to be a marker of poor prognosis in ALD if drinking continued [110, 118]. It has not been reported per se in NAFLD. Ultrastructural studies have shown myofibroblast proliferation around the terminal hepatic venule, in association with perivenular fibrosis [118]. Therefore, this lesion is closely linked to perisinusoidal fibrosis, a "chicken wire" pericellular scarring pattern of sinusoidal wall characteristic of steatohepatitis (both alcoholic and nonalcoholic). Indeed, zone 3 perisinusoidal fibrosis has been well documented in the early stages of alcohol liver injury combined with the thickening of centrilobular veins (phlebosclerosis) to produce dense stellate zones of centrilobular scarring flanked by ongoing hepatocyte injury and inflammation of varying degree [117].

Acinar zone 3 perivenular and perisinusoidal fibrosis is generally considered to be the main pathway for the subsequent development of bridging fibrosis and cirrhosis in ALD (Fig. 12.6). However, fibrosis involving the portal tract also contributes early to the development of fibrous septa, to the overall area of fibrosis, and to the subsequent lobular disarray [119]. These changes may include inflammation and ductular reaction, both of which contribute to the development of periportal fibrosis. It has been suggested that portal lymphocytic infiltration occurs as the predominant histological finding and leads to the development of periportal and septal fibrosis [120]. In this context, ductular reaction is common and may actively participate to the development of periportal fibrosis [121, 122]. The progression of zone 3 perisinusoidal fibrosis in association with periportal fibrosis predisposes to the development of bridging fibrosis. Initially, the developing fibrous septa are delicate and extend through sinusoids from terminal hepatic venules to adjacent hepatic venules (central-central bridging) or to portal regions (central-portal bridging). Portal tract to portal tract bridging is less frequent. Cirrhosis develops gradually over years of persistent injury.

The indolent progression of centrilobular fibrosis over many years in the steatotic form of alcoholic liver disease is to be contrasted with the aggressive progression of fibrosis in alcoholic hepatitis. The extensive hepatocellular damage and parenchymal inflammation that occur in

alcoholic hepatitis stimulate brisk fibrogenesis with formation of fibrous septa and cirrhosis. Indeed, in some cases, major perisinusoidal fibrosis may develop as a consequence of AAH with portal hypertension in the absence of septal fibrosis or cirrhosis.

The cirrhosis that can develop out of this environment constrains hepatocyte cords between collagen fibers, leaving little space for hepatocellular regeneration. It creates the characteristic "micronodular" pattern of cirrhosis in alcoholic liver disease (Fig. 12.6) [123]. Indeed, regenerative activity of entrapped hepatocytes is generally mild and generates remarkably uniform-sized small hepatocyte nodules, most of which are <3 mm in diameter or smaller. Micronodular cirrhosis is the most common type of cirrhosis seen in association with alcohol [123]. Bands of fibrous tissue are usually thick, made of densely packed collagen fibers that completely surround the hepatocyte nodules at the single lobule level; the terminal hepatic venules are not recognizable, but new shunting vessel formation is apparent within the fibrous tissue. As fibrous tissue develops, the liver loses fat and shrinks progressively in size. Parenchymal islands are engulfed by fibrous tissue with area of few residual hepatocytes leading to areas of parenchymal extinction frequently associated with ductular reaction. Ongoing ischemic necrosis contributes to fibrous obliteration of nodules. A variable mixture of neutrophils, lymphocytes, plasma cells, and macrophages is seen in the fibrous tissue [124]. When present, features of steatosis or AAH occur predominantly at the periphery of the regenerative nodules.

Subsequent remodeling of cirrhotic micronodules is typically observed after prolonged alcohol abstinence and may give rise to a mixed pattern of nodularity [125]. The regenerative nodules vary greatly in size, and some may measure up to several centimeters in diameter with portal tracts and terminal hepatic venules which are abnormally related to each other. A mixed micronodular and macronodular cirrhosis is a typical finding in explanted liver after long-term abstinence. In cases with less well-established cirrhosis, alcohol abstinence may result in considerable regression of fibrosis, resulting in incomplete fibrous septa without regenerative nodules (incomplete septal cirrhosis) [126].

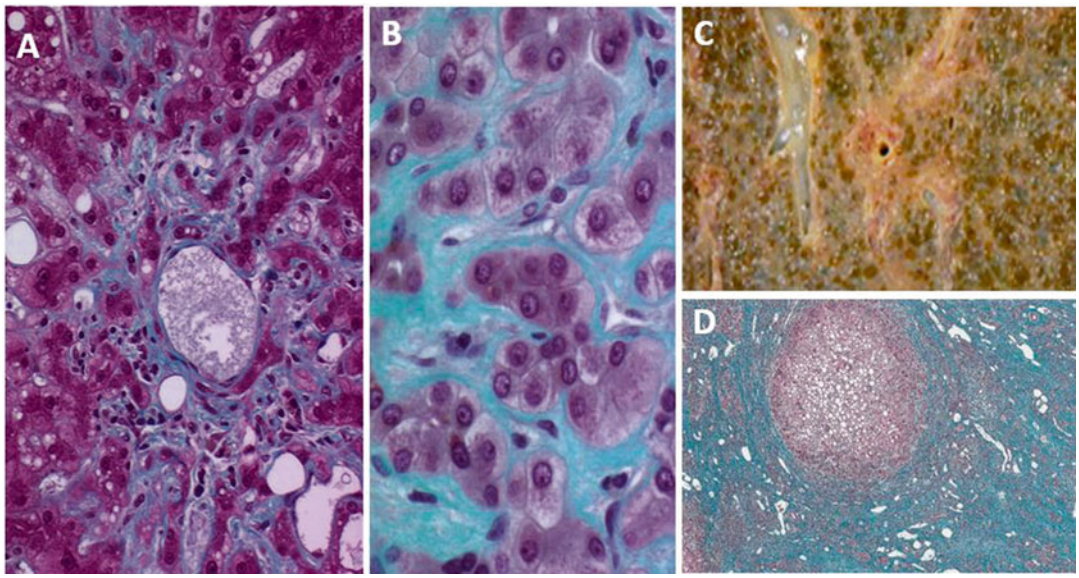

Fig. 12.6 Fibrosis patterns in alcoholic liver disease. (**a**) Masson trichrome stain highlights the acinar zone 3 fibrosis with few inflammatory cells. (**b**) Perisinusoidal deposit of dense fibrous tissue obstructing partially the sinusoidal lumen. (**c**) Gross section of micronodular alcoholic cirrhosis. (**d**) Alcoholic cirrhosis with thick fibrous tissue bands and small hepatocyte nodules

Hepatocellular Carcinoma and Cholangiocarcinoma

Studies from the USA and Italy suggest that in Western countries, alcohol may be the most common cause of HCC accounting for 32–45 % of cases [127]. The annual incidence of HCC is 1–2 % per year in alcoholic cirrhosis [128] and HCC develops in 5–15 % of patients with alcoholic cirrhosis, usually in association with macronodular cirrhosis. Development of HCC in absence of cirrhosis is a rare occurrence in ALD [129]. Autopsy studies in the past, as well as recent prospective studies of liver biopsies, have suggested that dysplastic nodules which develop within cirrhotic parenchyma may be the precursor lesion for hepatocellular carcinoma [130, 131].

Recent epidemiological studies have demonstrated an increased prevalence and incidence of intrahepatic cholangiocarcinoma and a relationship to the presence of cirrhosis [132]. Interestingly Wu et al. recently described biliary intraepithelial neoplasia lesions in the livers of patients with alcoholic cirrhosis suggesting that these may be precursor lesions for the development of biliary malignancy [133].

Other Features

A number of other features may be identified in biopsies of ALD. Periodic acid–Schiff-positive diastase-resistant globules resembling alpha-1 antitrypsin globules are frequently seen in hepatocytes at the periphery of cirrhotic nodules; this accumulation is considered to be a consequence of impaired protein secretion, whereas some globules stain immunohistochemically as alpha-1 antitrypsin [134]. Ground-glass changes in the cytoplasm of hepatocytes may occur in chronic alcohol abusers. They have been ascribed to proliferation of smooth endoplasmic reticulum related to alcohol-induced upregulation of drug-metabolizing enzymes [135]. Hepatocytes may also undergo oncocytic change, characterized by an intensely eosinophilic granular cytoplasm resulting from increased numbers of mitochondria [136].

Excess stainable iron is found in both hepatocytes and Kupffer cells in many patients with alcoholic liver disease [137]. Siderosis is usually mild. Possible mechanisms for the excessive iron accumulation in ALD include upregulation of the transferrin receptor and intestinal absorption, high iron content of some alcoholic beverages, and hemolysis associated with spur cells leading to increased iron absorption [138–141]. In alcoholic siderosis, the iron-containing hepatocytes are distributed in a random fashion and the iron granules are often few. More severe siderosis is seen in alcoholic cirrhosis [142, 143]. Parenchymal siderosis tends to have a zonal distribution, being most prominent in periportal/periseptal regions and can thus resemble changes seen in genetic hemochromatosis.

Alcohol is thought to hasten the onset of the hepatic and cutaneous manifestations of porphyria cutanea tarda; alcohol withdrawal is followed by a dramatic clinical and biochemical improvement. The hepatocytes may contain needle-shaped cytoplasmic inclusions of uroporphyrin which show brilliant-red autofluorescence under ultraviolet light; this is specific for porphyria cutanea tarda. Variable degrees of siderosis are usually present in the liver, and in addition, there may be evidence of alcoholic liver disease [144].

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13 Hepatic and Extrahepatic Malignancies in Alcoholic Liver Disease

Samir Zakhari, Svetlana Radaeva, and Vasilis Vasiliou

Introduction

While epidemiological studies point to an increased risk of various cancers associated with heavy alcohol consumption (cancers of the upper aerodigestive tract [oral cavity, pharynx, larynx, and esophagus], colorectum, liver, and female breast) and decreased risk of other cancers (renal cancer and non-Hodgkin's lymphoma), these studies cannot determine cause and effect. While heavy drinking may be linked to certain cancers, the ultimate outcome could be due to alcohol consumption alone, to its interactions with other lifestyle factors such as smoking, or to the presence of comorbid conditions such as obesity or viral hepatitis. Various cancers are tissue specific and comprise a multistage and complex process characterized by molecular alterations that underlie their initiation, promotion, and progression over a long time. Many hypotheses have been spawned to explain plausible mechanisms by which heavy drinking may be linked to carcinogenesis. Chronic heavy ethanol (hereinafter referred to as alcohol) consumption is a risk factor for cancer of the esophagus and oral cavity and an etiological factor in liver cancer [1]. This article focuses mainly on alcohol-associated hepatocellular carcinoma (HCC) and briefly discusses other cancers attributed to heavy alcohol consumption.

Hepatocellular carcinoma comprises approximately 85 % of primary liver cancer and is the third leading cause of cancer deaths worldwide. In 2012, it resulted in approximately ¾ million deaths globally [2]. HCC usually occurs as a consequence of chronically damaged livers due to cirrhosis, chronic infection with hepatitis B (HBV) and C (HCV) viruses, chronic heavy alcohol consumption, or cirrhosis associated with nonalcoholic steatohepatitis and primary hemochromatosis [3]. Other established causes of liver cancer include contraceptives high in estrogen and progesterone, smoking, obesity, and ingestion of food contaminated with fungal aflatoxin in subtropical regions.

Hepatocellular Carcinoma

While moderate alcohol consumption by adults could be a part of a healthy lifestyle for a large segment of the population [4], chronic heavy

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drinking invariably results in a spectrum of alcoholic liver disease (ALD) ranging from fatty liver (steatosis) in the majority of excessive drinkers to steatohepatitis and fibrosis [5] in about 35 % and only about 10 % progress to cirrhosis [6]. Among patients with cirrhosis, about 1–2 % develops HCC [7]. In addition, heavy alcohol consumption may act synergistically with HBV or HCV infection or obesity to induce HCC. These factors have some common features/mechanisms that exacerbate liver damage when they coexist [8]. The molecular aberrations in HCC pathogenesis are elegantly reviewed elsewhere [9, 10].

Mechanisms of Alcohol-Induced Hepatocarcinogenesis

Alcohol Metabolism

Ingested alcohol is readily absorbed from the gastrointestinal tract. Over 90 % of absorbed alcohol is metabolized mainly by oxidative pathways in the liver and to a small extent by non-oxidative pathways in extrahepatic tissues. Although alcohol metabolism is often considered a predominant factor in causing alcohol-associated liver damage, other factors, such as inflammatory cytokines, immunologic and metabolic pathway derangements, effects on signal transduction, proteasome inhibition, increased gut leakiness and LPS absorption, activation of Kupffer and hepatic stellate cells, genetic and epigenetic factors, etc., contribute to ALD.

The major pathway of oxidative metabolism of alcohol in the liver involves multiple isoforms of cytosolic alcohol dehydrogenase (ADH), which results in the production of acetaldehyde. The cytochrome P450 isozymes, including CYP2E1, 1A2, and 3A4, which are predominantly localized to the endoplasmic reticulum (ER), also contribute to alcohol's oxidation to acetaldehyde in the liver. CYP2E1 is induced by chronic alcohol consumption and assumes an important role in metabolizing alcohol to acetaldehyde at elevated alcohol concentration. Accumulation of acetaldehyde, a highly reactive and toxic molecule, contributes to liver damage. The oxidation of alcohol is accompanied by the reduction of NAD^+ to NADH and, thereby, generates a highly reduced cytosolic environment in hepatocytes. It also produces highly reactive oxygen species (ROS), including hydroxyethyl, superoxide anion, and hydroxyl radicals. Another enzyme, catalase, located in peroxisomes, is capable of oxidizing alcohol in the presence of a hydrogen peroxide (H_2O_2)-generating system, such as NADPH oxidase or xanthine oxidase. Quantitatively, however, this is considered a minor pathway of alcohol oxidation.

Acetaldehyde, produced by alcohol oxidation through any of these enzymes, is rapidly metabolized mainly by mitochondrial aldehyde dehydrogenase (ALDH2), and to a small extent by cytosolic aldehyde dehydrogenase (ALDH1), to form acetate and NADH. Mitochondrial NADH is reoxidized by the electron transport chain. Most of the acetate resulting from alcohol metabolism escapes the liver into the bloodstream and is eventually metabolized to CO_2 by way of the tricarboxylic acid (TCA) cycle in cells with mitochondria capable of converting acetate to the metabolically active intermediate acetyl-CoA. This occurs primarily in tissues such as heart, skeletal muscle, and brain (Fig. 13.1).

Consequences of Alcohol Metabolism by Oxidative Pathways: Cancer Implication

Alcohol metabolism in the liver results in various products/effects with implications for hepatocarcinogenesis.

Acetaldehyde Generation/Adduct Formation

Acetaldehyde produced by alcohol oxidation, if accumulated to appreciable concentrations, can form adducts with DNA and RNA and decrease DNA repair. Acetaldehyde also has the capacity to react with lysine residues on proteins including enzymes, microsomal proteins, and microtubules and affect their function. Formation of protein adducts in hepatocytes may contribute to impaired protein secretion, resulting in hepatomegaly. In addition, there is evidence that acetaldehyde and malondialdehyde (a by-product of lipid peroxidation) can combine and react with lysine residues on proteins, giving rise to stable malondialdehyde–acetaldehyde (MAA) protein

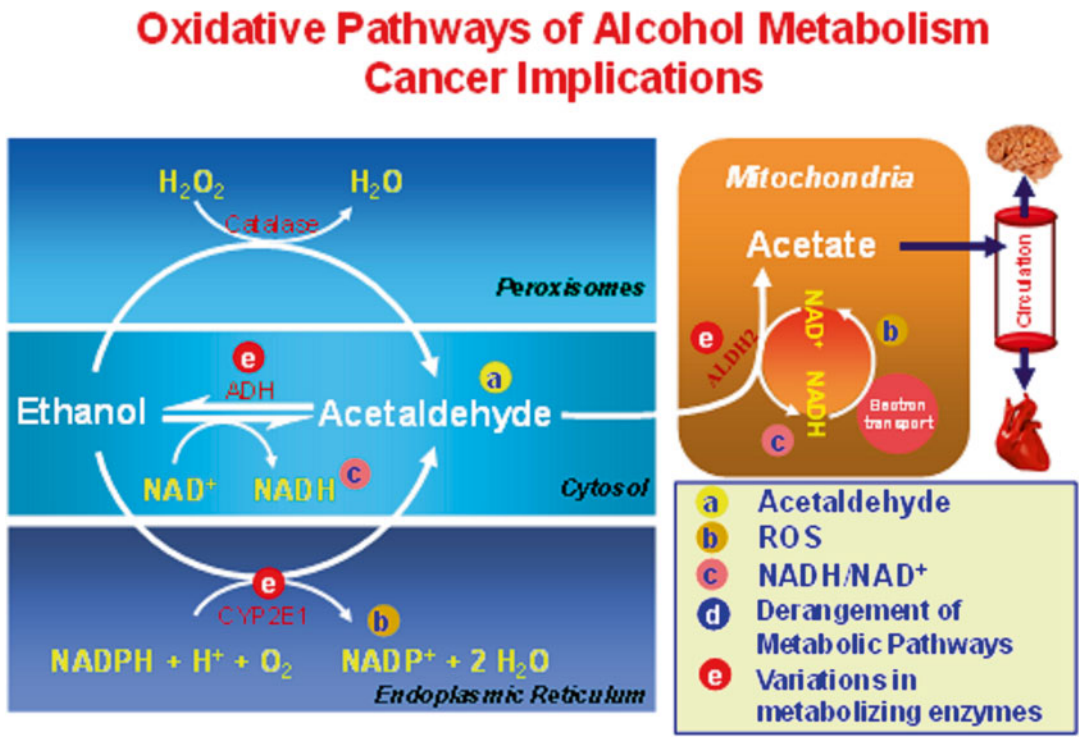

Fig. 13.1 Oxidative pathways of ethanol metabolism. *ADH* alcohol dehydrogenase, *ALDH* aldehyde dehydrogenase, *NAD* nicotinamide adenine dinucleotide, *NADH* reduced NAD, *NADP* nicotinamide adenine dinucleotide phosphate, H_2O_2 hydrogen peroxide, *ROS* reactive oxygen species

adducts that can be immunogenic and, thus, can contribute to immune-mediated liver damage. Also, MAA adducts have proinflammatory and profibrogenic properties.

The most relevant acetaldehyde adducts that impact the genome function and have implications to carcinogenesis are their interaction with the exocyclic amino group of deoxyguanosine to form DNA adducts (Fig. 13.2). These adducts involve the reaction of one molecule of acetaldehyde with DNA to form N2-ethylidenedeoxyguanosine, which is relatively unstable and abundant in human liver even in the absence of exogenous acetaldehyde [11]. This adduct is reduced in vivo with glutathione or vitamin C to form the stable N^2-ethyldeoxyguanosine (Et-dG). In addition, two molecules of acetaldehyde, or crotonaldehyde, form an adduct known as N^2-propano-2′-deoxyguanosine [12], which is maintained at a low steady state by DNA repair. A secondary acetaldehyde-related DNA adduct, 1,N^2-etheno-dG, is formed from acetaldehyde-stimulated lipid peroxidation. For a detailed discussion of these adducts and their genotoxic effects, the reader is referred to the review article by Brooks and Zakhari [13].

It should be noted that some results obtained from ADH1B polymorphisms do not concord with the acetaldehyde hypothesis. For example, a decreased UADT cancer risk was observed in drinkers who carried the ADH1B*2 allele that codes for the more active enzyme, leading to high acetaldehyde exposure [14].

Formation of Reactive Oxygen Species, Reactive Nitrogen Species, and Oxidative Stress

Hepatic mitochondria produce ROS through the activity of the electron transport chain (ETC) as a by-product of oxidative phosphorylation. Normally, a small fraction of electrons entering the ETC can prematurely escape from complexes I and III and directly react with ~1–3 % of respiratory oxygen molecules to generate the superoxide

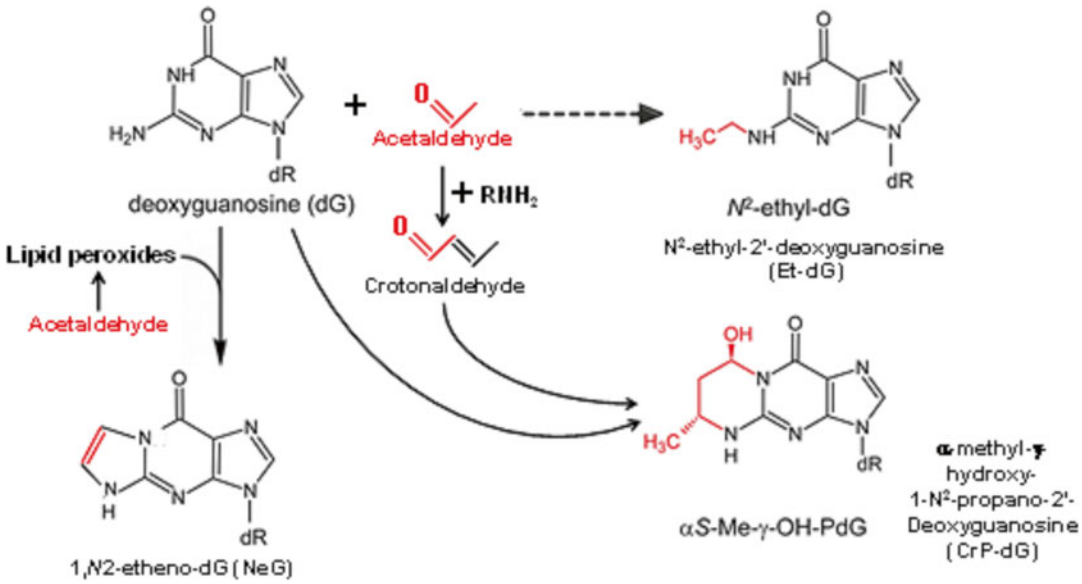

Fig. 13.2 Acetaldehyde: DNA adduct formation

anion radical, which is then dismutated by the mitochondrial manganese superoxide dismutase (MnSOD) into hydrogen peroxide (H_2O_2). Mitochondrial glutathione peroxidase (GPx) then converts H_2O_2 into water by using reduced glutathione (GSH) as a cofactor. Thus, most of the ROS generated by the ETC in the normal state are detoxified by the mitochondrial antioxidant defenses. The non-detoxified portion of ROS diffuses out of mitochondria and affects signal transduction pathways and gene expression, triggering cytokines, hormones, and growth factors, which if excessive may lead to hepatic inflammation, necrosis, and/or apoptosis. In addition, metals (e.g., iron and copper) can further react with H_2O_2 to produce hydroxyl radicals via the Fenton reaction. Nitric oxide (NO), a reactive nitrogen species critical for hepatocyte biology, can interact with peroxides to generate peroxynitrite ($ONOO^-$), which, depending on the amount and duration, could be detrimental to the liver (Table 13.1). NO is produced from L-arginine and oxygen by iNOS, which is expressed in all liver cells (hepatocytes, stellate cells, Kupffer cells, and vascular endothelial cells), and its expression is induced by IL-1β alone or in combination with TNF-α, IFNγ, and/or LPS. Alcohol not only produces ROS and reactive nitrogen species (RNS), but also depletes antioxidants in cells resulting in "oxidative stress." This condition regulates both genetic and epigenetic cascades underlying altered gene expression in human cancer [15].

Table 13.1 Chemical equations relevant to reactive oxygen and reactive nitrogen species generation

Reactive oxygen species generation
$O_2 + e^- \rightarrow O2^{-\bullet}$ (superoxide anion)
$O_2^- + H_2O \rightarrow HO_2^\bullet$ (hydroperoxyl radical)
$HO_2^\bullet + e^- + H \rightarrow H_2O_2$ (hydrogen peroxide)
$H_2O_2 + e^- \rightarrow OH^- + {}^\bullet OH$ (hydroxyl radical)
Reactive nitrogen species generation
L-arginine + $O_2 \rightarrow {}^\bullet NO$ (nitric oxide) + L-citrulline
$O2^{-\bullet} + {}^\bullet NO \rightarrow ONOO^-$ (peroxynitrite)
Fenton reaction (catalyzed by transition metals)
$H_2O_2 + Fe^{2+} \rightarrow OH^- + {}^\bullet OH + Fe^{3+}$
Haber–Weiss reaction (catalyzed by transition metals)
$H_2O_2 + O_2^{-\bullet} \rightarrow O_2 + {}^\bullet OH + OH^-$

It has been suggested that ROS participate in tumor progression by promoting DNA damage and/or altering cellular signaling pathways [16]. A reliable biomarker of oxidative stress and ROS-induced carcinogenesis is 8-oxo-7,8-dihydroguanine (8-oxoGua), which is strongly implicated in all stages of carcinogenesis [17].

Formation of 8-oxoGua lesions has been shown to induce DNA base mutations in the TP53 tumor suppressor gene in liver cancer cells [18]. Elevated levels of 8-OHdG in transgenic mice infected with HBV can lead to development of hepatocellular carcinoma [19]. In addition, oxidative stress often renders repair mechanisms ineffective. ROS have been reported to promote hypermethylation of the promoter region of the tumor suppressor E-cadherin, a regulator of the epithelial-to-mesenchymal transition, in HCC cells [20]. ROS induce hypermethylation of the E-cadherin promoter by increasing Snail expression. Alcohol promotes breast and colon cancer progression through stimulating the EMT program via a Snail-mediated pathway [21] and may have a similar effect in HCC. Exacerbating oxidative stress in livers infected with HCV by ROS induction and by hampering the antioxidant system facilitates hepatocarcinogenesis [22]. Ironically, increases in Nrf2 protein, a transcription factor that regulates important antioxidant and phase II detoxification genes, were observed in hepatocytes of alcohol-fed mice, suggesting that Nrf2 plays a key role in the adaptive response against increased oxidative stress caused by CYP2E1 [23].

Increase in NADH/NAD$^+$ Ratio

Alcohol metabolism produces a significant increase in the hepatic NADH/NAD$^+$ ratio in both the cytosol and the mitochondria, as evidenced by an increase in the lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios, respectively [24] Consequently, alcohol oxidation vastly increases the availability of oxidizable NADH to the electron transport chain in the mitochondria. The liver responds to alcohol exposure in part by increasing the rate of oxygen uptake, which may lead to periods of hypoxia, particularly in the downstream (pericentral) parts of the liver lobule. Increased NADH/NAD+ ratio provides reducing equivalents and thus enhances the activity of the respiratory chain, including heightened oxygen use and ROS formation [25]. Furthermore, the increase in the NADH/NAD$^+$ ratio results in derangement of carbohydrate metabolism and modulation of gene expression of, among others, SIRT1, a NAD$^+$-dependent deacetylase [26] whose substrates include histones and the transcription factor p53 [27]. Increased NADH in hepatocytes due to alcohol metabolism may promote tumor growth by favoring the generation of lactate through a NADH-dependent enzyme, lactate dehydrogenase A (LDH-A), which catalyzes the conversion of pyruvate to lactate during glycolysis. Tumor cells utilize more glucose than normal tissue, favor aerobic glycolysis, and rely on lactate production for their survival. A molecular mechanism underlying the enhanced lactate production in cancer cells involves tyrosine phosphorylation which enhances LDH-A enzyme activity to promote tumor growth by regulating the NADH/NAD redox homeostasis [28].

Derangement of Metabolic Pathways

Increased NADH/NAD$^+$ ratios in both the cytosol and mitochondria of hepatocytes influence the direction of several reversible reactions leading to alterations in hepatic lipid, carbohydrate, protein, lactate, and uric acid metabolism. These changes include (1) ***alcoholic hypoglycemia***, the increase in NADH prevents pyruvate conversion to glucose by lowering the concentration of pyruvate, which in turn decreases the pyruvate carboxylase reaction, one of the rate-limiting steps of gluconeogenesis; (2) ***hampering of the tricarboxylic acid (TCA) cycle function***, the increase in mitochondrial NADH in hepatocytes contributes to the saturation of NADH dehydrogenase; and (3) ***alcoholic acidosis***, ketoacidosis is common in chronically malnourished alcoholics and is due to the formation of ketone bodies, primarily β-hydroxybutyrate [29]. In addition, the increase in NADH favors the conversion of pyruvate to lactate, resulting in lactic acidosis. The increase in NADH/NAD$^+$ ratio diminishes pyruvate dehydrogenase (PDH) activity in the mitochondria, resulting in diminished conversion of pyruvate to acetyl-CoA. PDH activity is further diminished in chronic alcoholics due to hypomagnesemia and thiamine deficiency, resulting in the inhibition of pyruvate utilization in the TCA cycle; (4) ***hypoxia***, alcohol metabolism by hepatocytes tends to increase oxygen uptake,

resulting in significant hypoxia in the perivenous hepatocytes, the site of early liver damage due to chronic alcohol consumption.

Perhaps the derangement most relevant to HCC is alcohol impairment of retinoic acid (RA) synthesis and transport. Alcohol dramatically changes vitamin A and RA availability [30] and thus can impact carcinogenesis [31]. RA deficiency in alcoholics results from poor dietary intake and decreased absorption of retinoids and the significant overlap in metabolic pathways of alcohol and retinol, the alcohol form of vitamin A. Alcohol and retinol can be oxidized by similar, and sometimes identical, enzymes. In addition to the competitive inhibition of RA biosynthesis, prolonged alcohol consumption decreases tissue RA concentrations by enhancing its catabolism through the induction of cytochrome P450 enzymes and increasing mobilization of retinoids from the liver to extrahepatic tissues [32]. As a result, alcohol profoundly depletes hepatic retinoids and alters their distribution in other tissues [33, 34]. In addition to directly affecting RA metabolism and transport, alcohol may also affect plasma retinol concentration and its organ distribution indirectly through LPS-induced inflammation that reduces the level of RBP mRNA in the liver resulting in the impairment of the transport of retinol from the liver to plasma [35].

Changes in RA availability due to dysregulation of retinoid transport by alcohol are also becoming more evident [1]. It is strongly linked to alterations in differentiation/proliferation status of hepatocytes.

RA acts as a signaling molecule and regulates gene expression by binding to two subclasses of nuclear receptors, retinoic acid receptors (RARα, β, and γ isotypes) and retinoid X receptors (RXRα, β, and γ isotypes), encoded by distinct genes [36]. Alcohol affects the expression and activation of RA receptors, which in turn can impair the signaling events and induce harmful effects on cell survival and differentiation [37]. Recent developments indicate that alcohol can contribute to the aberrancy of retinoid nuclear receptor function and increased risk of cancer development through epigenetic alterations [38]. Alterations in the level of expression or functional activity of retinoid nuclear receptors are associated with a variety of cancers despite normal vitamin A levels. Hepatic retinoid level reduction by alcohol can lead to enhanced fibrogenesis that, in turn, may eventually constitute an irreversible process with regenerative diffuse parenchymal nodular transformation, cirrhosis, and HCC. Alcohol-related HCCs are associated with cirrhosis in a majority of cases, indicating that the pathological events leading to cirrhosis precede those causing cancer or that the structural alterations of cirrhosis favor hepatocyte dedifferentiation [39]. Since dedifferentiation is ultimately associated with increased proliferation rate, the dedifferentiation hypothesis fits perfectly with findings that low hepatic RA concentration due to alcohol leads to an upregulation of AP-1 (c-jun and c-fos) and beta-catenin-dependent gene expression that may promote proliferation and malignant transformation of hepatocytes by alcohol [34, 40]. Interestingly, alcohol-induced RA-dependent hepatocyte hyperproliferation may not only lead to the neoplastic transformation of preexisting hepatocytes but may also compromise organ regeneration by liver stem cells. The activation of liver stem cells requires a uniform inhibition of parenchymal proliferation [41] and their differentiation depends on RA [42, 43]. It is interesting to note that liver stem cells are highly responsive to vitamin A deprivation [44] and always appear in close proximity to activated hepatic stellate cells (HSC) suggesting a possible involvement of RA in control of their behavior. Deficiency of RA in alcoholic livers blocks differentiation and apoptosis in the progeny of liver stem cells, while promoting their proliferation. This may explain the development of anaplastic poorly differentiated HCCs without preexisting cirrhosis, which are also observed in alcoholics, albeit rarely [45, 46].

Variations in Metabolic Enzymes

Class I ADH and ALDH2 play a central role in alcohol metabolism. Allelic variations in the genes encoding ADH and ALDH produce alcohol- and acetaldehyde-metabolizing enzymes that vary in activity. These genotypes modify the

susceptibility to tissue damage. The ADH gene family encodes for enzymes that metabolize various substrates, including retinol, and are differentially expressed in different organs. This highlights the important issue of substrate competition in alcohol-induced tissue damage. Genetic polymorphism occurs at the ADH1B and ADH1C loci [47] with different catalytic activities for alcohol. The ADH1B alleles occur at different frequencies in different populations. For example, the ADH1B*1 form is found predominantly in Caucasian and Black populations, while ADH1B*2 frequency is higher in Chinese and Japanese populations and in 25 % of people with Jewish ancestry. A significant interaction exists between ADH1B polymorphism and heavy alcohol consumption especially for those with ADH1B*1/*1 genotype and esophageal [48] and UADT [49] cancer. Several isozymes of ALDH have been identified, but only the cytosolic ALDH1 and the mitochondrial ALDH2 metabolize acetaldehyde. There is one significant genetic polymorphism of the ALDH2 gene, resulting in allelic variants ALDH2*1 and ALDH2*2 (glutamine to lysine substitution at position 487, resulting in 100-fold increase in the Km for NAD^+, making the gene product virtually inactive). The low activity ALDH2*2 is a deficient phenotype, which is present in about 50 % of the Taiwanese, Han Chinese, and Japanese populations [50] and shows virtually no acetaldehyde-metabolizing activity in vitro. The activity of ADH and ALDH isozymes contributes to alcohol-induced tissue damage. Alcoholic cirrhosis is reduced over 70 % in populations carrying the ALDH2*2 allele [51]. The activities of class I ADH are much higher in cancerous than in healthy tissues [52], and individuals with the ADH1C*1 allele have an increased risk to develop breast cancer from alcohol [53]. Furthermore, ALDH2-deficient individuals are at much higher risk of esophageal cancer (specifically squamous cell carcinoma) from alcohol consumption than individuals with fully active ALDH2 [54]. The correlation between genetic polymorphism of ADH and ALDH and esophageal, head, and neck cancers was reviewed by Yokoyama and Omori [55] and summarized in Table 13.2.

Although several CYP2E1 polymorphisms have been identified, only a few studies were undertaken to determine the effect on alcohol metabolism and tissue damage. In one study, the presence of the rare c2 allele was associated with higher alcohol metabolism in Japanese alcoholics but only at high blood alcohol concentrations of 0.25 g/dL [56]. In addition, induction of CYP2E1 also contributes to carcinogenesis through activation of pro-carcinogens, such as nitrosamines present in diets and in tobacco smoke, to their carcinogenic metabolites [57]. The correlations between genetic polymorphisms and risk of alcohol-related cancers are reviewed elsewhere [58].

Epigenetic Modifications

Functional genomic studies (GWAS, whole-genome sequencing, global DNA copy numbers and methylation, and gene or noncoding RNA expression profiling) revealed that genetic polymorphisms of immune-related genes, such as

Table 13.2 Alcohol, genetic polymorphism, and cancer

Population	Cancer type	Genotype	Result	Reference
Asian	UADT	*ADH1B*1* (slow)	Increased risk	[55, 58]
Asian	UADT	ALDH2*1/*2	Increased risk	[49, 157]
Asian	Esophageal	*ADH1B*1/*1*	Increased risk	[48]
Japanese	Esophageal	*ALDH2*1/*2*	Increased risk	[158]
European	Various	*ADH1B*2* (fast)	Protective	Hashibe et al. (2006)
Caucasians	Head and neck	*ADHIC*1* (fast)	Increased risk	[159, 160]
European	Head and neck	*ADHIC*2* (slow)	Increased risk	Hashibe et al. (2006)
Caucasian	Head and neck	*ADHIC*2* (slow)	Increased risk	[161, 162]
Japanese	Head and neck	*ALDH2*1/2*	Increased risk	[163]

IL28B and MHC class I and II molecules (e.g., MICA), and somatic mutations of TP53 and ARID2 (a novel liver cancer-related gene that encodes a component of the SWI/SNF chromatin-remodeling complex) as well as activated β-catenin mutations are associated with HCC initiation and progression [59]. In addition, epigenetic mechanisms play an important role during the development and progression of HCC, and numerous studies have identified a large number of genes and pathways that are subject to epigenetic dysregulation [60]. Global DNA hypomethylation, histone modifications, promoter methylation, aberrant expression of noncoding RNAs, and dysregulated expression of epigenetic regulatory genes such as EZH2 are the best-known epigenetic abnormalities [61]. As mentioned above, alcohol metabolism alters the ratio of NAD^+ to NADH and promotes the formation of ROS and acetate, all of which impact epigenetic regulatory mechanisms. Furthermore, the activities of enzymes involved in epigenetic modifications, such as DNA and histone methylation and histone acetylation, are influenced by the levels of metabolites such as NAD^+, adenosine triphosphate (ATP), and S-adenosylmethionine (SAM). Chronic alcohol consumption leads to significant reductions in SAM levels, thereby contributing to DNA hypomethylation. These epigenetic changes are discussed in detail elsewhere [62] and are briefly mentioned below.

Epigenetic Effects

Since only about 10 % of heavy drinkers develop cirrhosis, the complex biological processes underlying states of health and disease could be determined by interactions between many genes and the external environment, and are likely to be driven by both genetic defects and by modifications that affect the transcriptional capacity of these genes (epigenetics). Epigenetic changes (e.g., DNA methylation or histone modification) affect gene expression directly or through the way DNA is packaged into chromatin, thus altering accessibility to transcription factors. In general, methylation of DNA represses gene expression by changing the chromatin structure or by interfering with the binding of some transcription factors to the promoter. DNA methylation has been shown to play a critical role in many cellular and biological processes, including cancer, aging, development, and the maintenance and differentiation of stem cells [63]. The successful establishment and maintenance of transcriptional profiles depend on the interplay between epigenetic modifications, interacting proteins, noncoding RNAs, and inter- and intrachromosomal interactions. Perturbation of any one of these regulatory elements may have profound consequences on the liver.

Chronic alcohol consumption has been shown to affect epigenetic regulation of gene expression involving DNA methylation, histone modification, and RNA-mediated gene silencing, thus modulating the expression of many genes. Alcohol-induced changes in gene expression may affect various biochemical and signaling pathways influencing the function of cells and organs, leading to liver disease and even cancer. Understanding alcohol-induced epigenetic regulation will provide mechanistic insights, diagnostic biomarkers, and therapeutic targets for alcohol-related liver injury.

Alcohol and DNA Methylation

DNA methylation tags cytosine, one of the four chemical bases that make up the genetic code, with a methyl group by transferring a methyl group from SAM onto the cytosine residue, which protrudes into the major groove of the DNA. Although acetaldehyde, the first metabolite of alcohol, can form DNA adducts and may cause sequence alteration of DNA, most of the short or long-lasting effects of alcohol may be independent of DNA sequence changes. Recent studies have shown that epigenetic regulation of gene expression is an important mechanism for alcohol's action in the cell and alcohol-induced liver damage [38].

Alcohol-induced epigenetic changes may also affect stem cell differentiation and liver repair and regeneration. These processes are characterized by rapid, well-synchronized patterns of gene expression in which the status of DNA methylation shifts dramatically, involving both loss of methylation and de novo methylation. Many epigenetic changes during stem cell differentiation

involve genes known to function in cell cycle, growth, apoptosis, and oxidative stress, all of which play a critical role in alcohol-induced liver damage and cancer. Evidently, purely sequence-based genetic or genomic approaches to study gene regulation are not sufficient to explain alcohol-related HCC.

Effects of Alcohol on the Availability and Transfer of Methyl Groups

Chronic alcohol consumption is associated with abnormal methionine metabolism, increased plasma homocysteine level, decreased level of SAM, and folate deficiency [5, 64]. SAM, the major methyl donor for DNA methylation, is primarily generated in the liver from L-methionine and ATP by methionine adenosyltransferase (MAT), which is encoded by two genes, *MAT1A* and *MAT2A*. *MAT1A* encodes the isoenzymes MATI and MATIII, whereas *MAT2A* gene encodes the isoenzyme MATII. MATI and MATIII are primarily responsible for maintaining high intracellular SAM levels in adult liver, while MATII is predominantly active in fetal and regenerating liver tissues.

Alcohol impairs the transfer of methyl groups to the cytosine residues of DNA by reducing the levels and activity of DNA methyltransferases (DNMT), resulting in DNA hypomethylation. The alcohol metabolite acetaldehyde can also inhibit DNMT activity. Studies have shown that livers of *MAT1A* knockout mice had SAM deficiency and increased expression of genes involved in proliferation and consequently developed hepatomegaly, fatty liver, and eventually HCC. They also regenerated abnormally after partial hepatectomy and were more sensitive to developing steatosis in response to a methionine- and choline-deficient diet [65]. It has been reported that during hepatocarcinogenesis or hepatectomy, *MAT1A* itself is severely downregulated due to the hypermethylation of its promoter [5]. These observations suggest that alcohol may contribute to HCC development via its inhibition of MATI and MATIII activity as well as reducing SAM levels [66].

In addition to its effects on MAT and SAM synthesis, alcohol also inhibits a number of methyl group transfer-related enzymes such as methionine synthase and cystathionine-β-synthase. The latter removes homocysteine through trans-sulfuration to cystathionine, which is used to generate the antioxidant glutathione (GSH). The net result is a decreased level of GSH, leading to increased oxidative stress, also contributing to liver damage. Excessive alcohol intake can decrease the GSH level by inhibiting hepatic GSH synthesis and the enzymatic activities involved in GSH-related peroxide detoxification such as GSH peroxidase and glutathione S-transferase (GST), thus increasing the susceptibility of the liver to oxidative injury [67].

Another major site of alcohol's actions on methyl group transfer is the folate metabolism cycle. Chronic alcohol consumption causes malabsorption of folates and increases their renal excretion resulting in a significant decrease in hepatic folate content [68]. In addition, it inhibits methionine synthase which transfers a methyl group from 5-methyl tetrahydrofolate to homocysteine to form methionine.

Although it is clear that chronic alcohol ingestion alters availability and transfer of methyl groups, its impact on DNA methylation appears to be complex. While DNA methylation in the promoter regions of class I ADH genes is elevated in an alcohol-treated human hepatoma cell line [69], the expression of DNA methyl transferase (DNMT-3b) is decreased in alcoholic patients [70]. The effect of alcohol on DNA methylation may depend on genomic context, cell type, and target organ.

Histone Modification

Chromatin structure is dynamically regulated to selectively facilitate the expression of some genes while maintaining others in a quiescent state and to allow for DNA repair and replication. Genes within highly condensed "heterochromatin" regions are generally silenced, whereas uncondensed "euchromatin" is permissive for gene expression. The various states of chromatin are largely attributable to the posttranslational modification of histones. These occur primarily on the N-terminal histone "tails" that protrude from the nucleosome structure and include

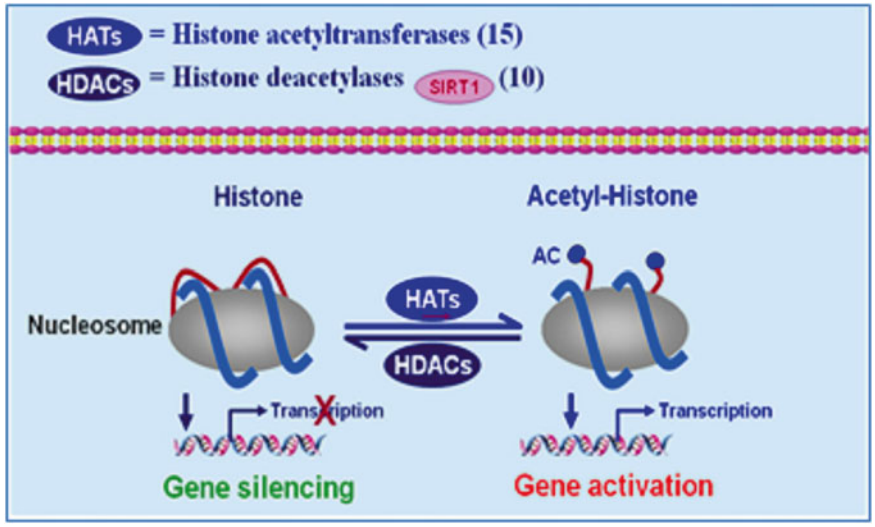

Fig. 13.3 Histone acetyltransferases (HATs) and deacetylases (HDACs) influence the acetylation, transcription, and condensation of chromatin

mono-, di-, or tri-lysine methylation, lysine acetylation, lysine ubiquitination, arginine methylation (mono- or di-), and serine or threonine phosphorylation, among others.

Histone acetylation is associated with loosely packed chromatin and actively transcribed genes. As depicted in Fig. 13.3, histone acetylation is determined by the opposing activities of histone acetyltransferases (HATs) and deacetylases (HDACs). Histone methylation results from the action of histone methyltransferases (HMTs) and is reversed by histone demethylases. Enzymes involved in histone acetylation are relatively few in number and promiscuous in terms of which lysines they modify. In contrast, HMTs and histone demethylases are typically specific for a single H3 or H4 residue and, consequently, are more numerous [71].

Alcohol and Histone Modification

Emerging evidence points to the potential of alcohol to exert its health effects by altering the state of chromatin. Acute alcohol administration to rats was shown to increase H3K9 acetylation in selected tissues, including liver, but not in others, indicating that epigenetic effects of alcohol will likely vary by tissue [72]. Consistent with these in vivo findings, in vitro studies found that alcohol also promotes the acetylation of H3K9 in primary hepatocyte cultures without affecting the acetylation status of other H3 lysines, including K14, K18, K23, or K27 [73]. In addition, this specific effect on H3K9 was associated with increased HAT activity by the acetate derived from alcohol metabolism. Accompanying this increase in H3K9 acetylation, there was an overall reduction of methylated H3K9 and a concomitant increase in methylated H3K4 in alcohol-treated hepatocyte cultures [74]. At the individual gene level, genes whose promoters exhibited this predominant pattern were found to be transcriptionally active, while those exhibiting the inverse pattern (i.e., increased H3K9me, decreased H3K4me) were silenced. Together, these findings highlight the potential for alcohol to alter patterns of histone modification and the expression of associated genes, raising the possibility that oncogenes and/or tumor suppressors might be among the affected genes and represent a mechanism by which alcohol contributes to HCC.

The decrease in NAD^+/NADH ratio due to alcohol metabolism has the potential to diminish the activity of NAD^+-dependent enzymes, including the SIRT family of histone deacetylases (HDACs). Indeed, inhibition of hepatic SIRT1 activity by alcohol was associated with an

increase in the acetylated active nuclear form of SREBP-1c in the livers of alcohol-fed mice leading to impairment of lipid metabolism [75].

MicroRNAs, Cancer, and Alcohol

MicroRNAs (miRNAs) often regulate the expression of cancer pathway components, including oncogenes and tumor suppressors. While miRNAs are often globally downregulated in human tumors [76], some miRNAs are frequently dysregulated in many types of cancer. The degree to which the expression of some miRNAs is altered is correlated with clinical or pathologic indicators of malignancy, offering opportunities for early detection and insights into early pathogenesis [77].

HCC is of particular relevance to alcohol and has been the subject of several miRNA profiling studies. Murakami et al. [78] identified three miRNAs that were overexpressed in HCC (miR-224, miR-18, and pre-miR-18) and five others that were under-expressed (miR-199a, miR-199a*, miR-200a, miR-125a, miR-195). Furthermore, they demonstrated the effectiveness of a signature, based on these changes, in distinguishing HCC and non-HCC cases and identified three miRNAs (miR-92, miR-20, and miR-18) whose expression was inversely correlated with the degree of HCC differentiation. Another study defined miRNA changes that allowed differentiation of HCC (increased miR-21, miR-10b, and miR-222) from benign hepatocellular adenomas (decreased miR-200c and miR-203) [79]. Importantly, this study also revealed specific miRNA markers of alcohol-related HCC (decreased miR-126) and HCC associated with hepatitis B viral exposure (increased miR-96). The molecular pathways regulated by miRNA in HCC are detailed in a review by Milazzo and colleagues [80].

Effect of Alcohol on miRNA Expression and Function

Given the breadth of processes that miRNAs are known to regulate, it is reasonable to expect that they will play significant roles in mediating the effects of alcohol, including cancer. Several recent reports have identified miRNAs whose levels are altered by alcohol and that mediate alcohol's ability to promote gut leakiness [81]. Chronic alcohol consumption increased miR-21 expression during liver regeneration in ethanol-fed rats [82] and enhanced miR-155 in macrophages via NF-6B, which contributed to the elevation in TNF-α production [83]. In addition, chronic alcohol feeding resulted in significant alteration in several miRNAs that regulate hepatic metabolism (miR-34a, miR-103, miR-107, and miR-122) [84], as well as downregulation of miR-199, which may contribute to HIF-1α augmentation [85]. In addition, large-scale miRNA screens indicate that alcohol alters the expression of 2–3 % of miRNAs (miR-320, miR-486, miR-705, and miR-1224 and a decreased expression for miR-27b, miR-214, miR-199a-3p, miR-182, miR-183, miR-200a, and miR-322) in murine models of alcohol-induced steatohepatitis [86]. These changes in miRNAs and their significance were reviewed elsewhere [87].

Circadian Rhythm Perturbation

The heterodimer of transcription factors CLOCK and BMAL1, which activates transcription of the period (*Per*) and cryptochrome (*Cry*) genes, comprises the centerpiece of the mammalian circadian network [88]. Their products PER and CRY interact to form the PER/CRY complex which translocates into the nucleus to inhibit CLOCK/BMAL1 transactivation, which in turn results in the repression of the *Per* and *Cry* genes. Release of PER/CRY complex through proteasome degradation can relieve repression and start the negative feedback loop again.

Despite the fact that there is no direct evidence connecting circadian rhythm disturbance to alcohol-induced HCC, dysfunctions of circadian rhythms are involved in many diseases that are known to be modulated by alcohol. The hypothesis that disturbance in circadian rhythms by alcohol may be involved in cancer is supported by the following research:

- Disturbance in circadian rhythm genes expression is a common feature in certain types of cancer, including HCC [89]. Many studies have suggested indirectly that circadian rhythms may play an important role in hepatocarcinogenesis. Key clock genes have been

found to be disrupted in HCC patients [90], demonstrating that HCC impacts the orchestrated circadian rhythm of liver cells. In addition, a long noncoding RNA (lncRNA), highly upregulated in liver cancer (HULC), has been reported to contribute to the perturbations in circadian rhythm of hepatoma cells [91]. Furthermore, a single functional polymorphism of one SNP rs2640908 in *PER3* gene was significantly associated with overall survival of HCC patients [92].

- Liver metabolism can be greatly affected by circadian rhythms and changes in feeding status. A large number of metabolic enzymes, such as CYP2E1, CYP3A4, CYP3A11, ADH, and ALDH—many of which are involved in alcohol metabolism—are regulated by the circadian clock [93]. Many other important metabolic pathways such as glycolysis, fatty-acid metabolism, cholesterol biosynthesis, and xenobiotic and intermediate metabolism are also under circadian regulation [94]. The rate-limiting steps of these metabolic pathways are often the target sites of circadian control. Because of their important roles in metabolism, mutations in the clock genes often cause metabolic disorders. For example, a mutation of the *Clock* gene caused mice to be hyperphagic and obese, exhibiting hyperlipidemia, hepatic steatosis, hyperglycemia, and hypoinsulinemia [95]. Conversely, circadian clocks can be entrained by various metabolism-related external cues, such as food intake and alcohol consumption. A high-fat diet in mice can change the expression of clock genes and clock-controlled genes [96], delay the circadian expression of adiponectin signaling components, and inhibit AMPK expression in mouse liver [97]. Furthermore, some nuclear receptors, such as PPARα, PPARγ, glucocorticoid receptor, RARα, and RXRα, have been directly connected to the key components of the circadian system [98] and have been also implicated in alcohol-induced tissue injury.
- Some biological pathways closely related to alcohol's actions are involved in, or affected by, circadian rhythms. The redox state of cells plays an important role in the function of the circadian rhythm. The presence of NADH or NADPH promotes the binding of the heterodimeric clock transcription factor complexes to DNA [99]. In addition, studies suggest that the histone deacetylase SIRT1 may be involved in the integration of circadian and metabolic transcription networks. It has been shown that SIRT1 interacts directly with CLOCK and deacetylates BMAL1 and PER2. Malondialdehyde, a marker of oxidative stress affected by alcohol, is found to exhibit circadian patterns of expression in mice liver [100]. The retinoic acid receptors RXR and RAR can interact with CLOCK and NPAS2 (a homolog of CLOCK), and these interactions can be increased 15-fold by retinoic acid [101]. Other consideration is that circadian rhythm disturbances lead to immunodeficiency [102], suppression of natural killer cell activity, and alteration in the T-helper 1/T-helper 2 cytokine balance, resulting in decreases in cellular immunity and tumor immune surveillance [103], all of which are affected by alcohol.
- Lipopolysaccharide (LPS), which is increased in blood after alcohol consumption, suppresses clock genes, suggesting that circadian rhythms play an important role in response to systemic inflammatory stimulation [104].

The relative contributions of disrupted circadian rhythm and circadian genes to cancer risk may be informative as to the pathogenesis of various cancers and new treatment interventions.

Immune Modification

The first line of host defense against HCC is innate immunity, including natural killer (NK) cells and T lymphocytes. The involvement of the immune system in HCC carcinogenesis has been previously proposed in clinical studies, where the percentage and absolute number of NK cells were decreased significantly during the development and progression of HCC [105]. Effective adaptive immune response depends on the antigen-specific activation of T and B cells. Increased activity of helper T cells, which promote inflammation, is associated with HCC [106], and chronic inflammation has been implicated in

the development of liver cancer in humans [107]. While the immunoglobulin protein family (CD28 and cytotoxic T-lymphocyte antigen-4) plays important roles in the control of T-cell responses against infection and cancer, tumors seem to have exploited these pathways to evade immune surveillance. Activation and proliferation of cytotoxic T lymphocytes is suppressed in individuals with HCC [108].

Recent studies suggested a role of the immune system in constitutional susceptibility to HCC. A genome-wide association study (GWAS), focusing on HCC, revealed that constitutional genetic variations are risk factors for HCC [109]. Three susceptibility loci have been strongly associated with HCC including the class II MHC complex, whose protein products present antigen to T-cell receptors and mediate immune surveillance (rs9267673, rs2647073, and rs3997872) resulting in an ineffective T-cell response. MHC class II molecules present antigen to CD4. Thus, genes involved in the immune response play a critical role in the development of HCC. Since only a subset of liver cirrhosis patients develop HCC, the transition from cirrhosis to HCC has been attributed to two SNPs whose allele frequencies differ significantly between HCC and cirrhosis (one lies in the PTEN homolog TPTE2, and the second variant lies within an intron of TPTE2, which encodes a homolog of the PTEN tumor suppressor protein [110]). Multiple SNP analysis showed that "antigen processing and presentation" emerged as the pathway with the strongest association with HCC. Thus, the T-cell repertoire of each individual plays a critical role in HCC susceptibility and that biological processes affecting T-cell maturation or immune surveillance may represent important etiologic mechanisms for the development of HCC in humans.

The NK (natural killer cells) are able to recognize and kill invading pathogens and cancer cells. This capability depends on the balance between activating (CD16, NKG2D, NKG2C, CD226, CD244, and the natural cytotoxicity receptors) and inhibitory (killer cell immunoglobulin-like receptors [KIRs], CD94/NKG2A, and leukocyte immunoglobulin-like receptor 1 [CD85], most of which recognize MHC class I molecules) receptor signaling, a complex process requiring several NK cell surface receptors acting synergistically. By inducing the upregulation of inhibitory receptors and downregulation of activating receptors on NK cells, cancer cells can become "invisible" to immune surveillance. Furthermore, NK cell dysfunction may promote the escape of tumor cells. Numerous studies have found a reduction in the proportion of NK cells in peripheral blood of HCC patients [111] and a decrease in the expression of NK cell-activating receptors during the development and progression of HCC [112].

Chronic alcohol consumption could have profound effect on both innate and adaptive immunity [113]. Immune function inhibition by alcohol could allow tumor evasion from immune surveillance and ultimately establishing tumor growth. NK cell activity was decreased in abstinent alcoholics compared to nondrinkers [114] and alcoholics show reduced numbers of T cells (including CD4+ T cells, CD8+ T cells) with alterations in their cytokine expression [115].

As mentioned earlier, antitumor defense requires both effective antigen presentation and the capacity of the T cells to respond to the antigen priming. Chronic alcohol impairs antigen presentation by dendritic cells and monocytes and damages the primary response of CD8+ cells, but not CD4+ response to specific antigen priming in mice [116]. In addition, CD8+ cells from the ethanol-fed mice exhibited poorer proliferation and poorer IFNγ production after priming. Thus, chronic alcohol consumption impairs the interactions between the antigen-presenting cells and the T cells [117] and causes intrinsic defects in the T cells themselves [99].

Neoangiogenesis

The hallmarks of hepatic circulation in liver cirrhosis are vasoconstriction, sinusoidal remodeling, angiogenesis, and venous thrombosis, which all contribute to increase hepatic vascular resistance and portal hypertension [118]. Angiogenesis is the result of two opposing processes regulated by proangiogenic factors (e.g., vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiopoietin, EGF, and PDGF, which

induce angiogenic signaling via RAS/RAF/MEK/ERK, mTOR, and Wnt signal transduction pathways) and inhibitory factors (thrombospondin [TSP] and angiostatin) [119]. Increased expression and secretion of VEGFA due to hypoxia (mediated by hypoxia-inducible factor 2-α) of cancer cells [120] induces endothelial cells' proliferation, migration, survival, and angiogenesis which promote tumor growth [121]. Normally, HCC displays active angiogenesis, which not only contributes to increased vascular resistance and portal hypertension, but also allows cancer cells to invade vessels and metastasize [122].

Other signaling pathways involved in hepatocarcinogenesis include phosphatidylinositol-3 kinase (PI3K)/AKT/mTOR), Wnt/β-catenin, insulin-like growth factor, and hepatocyte growth factor/c-MET [123]. Wnt/β-catenin pathway contributes to HCC formation by influencing cell adhesion and transcriptional activation of target genes such as c-myc and cyclin D [124]. In fact, β-catenin accumulation, a hallmark of the activated Wnt/FZ signaling, has been observed in 33–67 % of HCC tumors [125]. In addition, miR-610 was downregulated in human HCC, thus promoting HCC cell proliferation and tumorigenicity by activating Wnt/β-catenin signaling [126]. On the other hand, overexpression of miR-153 was able to promote β-catenin transcriptional activity, leading to cell-cycle progression, proliferation, and colony formation of HCC cells [127].

Although no studies have examined the relationship between alcohol consumption and HCC angiogenesis, other studies showed that this is a plausible hypothesis. In experimental animals, alcohol consumption (equivalent to ~2 drinks/day in humans) by immune-competent mice implanted with mouse melanoma cells resulted in an increase in VEGF transcript and protein levels, doubling of tumor volume, and enhanced microvascular density [128]. Further studies showed that alcohol intake enhances angiogenesis in a rat model of choroidal neovascularization [129]. Chronic alcohol consumption increased HCC angiogenesis, progression, and metastasis through NFκB-dependent VEGF and MCP-1 upregulation [130].

In addition, alcohol-induced oxidative stress and inflammation further amplify vasoconstriction and portal hypertension and the ensuing angiogenesis, a hallmark in tumor maintenance [131].

Breast Cancer

Breast cancer is a heterogeneous disease that encompasses more than 20 different subtypes and has a wide range of known risk factors involved in its development. Many of the primary risk factors for breast cancer are beyond women's control, such as aging, inherited changes in certain genes and family history of breast cancer, prenatal history (e.g., daughters born to mothers who used diethylstilbestrol (DES) during pregnancy), and reproductive parameters such as first full-term pregnancy, miscarriage, and abortion. However, modifiable lifestyle risk factors under women's control include dietary habits (consumption of polyunsaturated fats and excessive alcohol), smoking, exposure to radiation or synthetic estrogens, viral infection, physical inactivity, use of HRT, obesity, diabetes, breast implants, and even changes in circadian rhythm homeostasis, such as night shift work. The interactions between genetic susceptibility for breast cancer and the environmental factors add another layer of complexity to this picture.

Epidemiological studies on alcohol and breast cancer are inconsistent. For example, in one study [132], consumption of one or two drinks/day increased breast cancer risk by 40 %, whereas consumption of two or more drinks/day resulted in no increase in risk. For moderate drinking, one study [133] stated that drinking <1.5 drink/day was associated with a 42 % decrease in breast cancer risk, whereas another [134] reported that drinking 3–6 drinks/week was associated with a 15 % increase in risk. A meta-analysis [135] stated "the modest size of the association and variation in results across studies leave the causal role of alcohol in question." The variability in outcomes could be ascribed to: (1) the majority of the studies rely on self-report to determine the amount of alcoholic beverage consumed, which introduces "recall bias" into the studies that

makes the alcohol consumption variable notoriously inaccurate; (2) since breast cancer develops over a period of more than 20 years [136], the correlations between current or recent alcohol consumption and breast cancer cannot be determined in epidemiological studies that captures intake after clinical diagnosis; (3) epidemiological study rarely differentiates between specific subtypes of breast cancer—which are associated with unique risk factors and might have been preprogrammed at an early stage of the disease—and risk of alcohol consumption. Better standardization of study designs in assessing alcohol intake and timing of exposure may improve our understanding of the heterogeneity of results across studies [137].

Despite the challenge in understanding the epidemiological findings, several molecular mechanisms have been postulated [138] for alcohol-associated breast cancer, including formation of acetaldehyde and ROS, epigenetic effect through the folate cycle, and estrogen formation. For a comprehensive review of alcohol and breast cancer, the reader is referred to the article by Zakhari and Hoek [139].

Colon Cancer

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world with an estimated 1.24 million new cases each year [140]. In the United States, the annual incidence of CRC is about 148,300, with 56,600 deaths per year, and the lifetime risk in the general population is about 5–6 % [141]. It appears that chronic heavy consumption of alcohol may increase the relative risk for colon cancer.

In the colon, acetaldehyde is primarily produced from ethanol by resident bacteria and, to a lesser extent, by mucosal ADHs. Human colon mucosal cells harbor ADH1, ADH3, and ADH5, with the ADH1 and ADH3 isozymes being most active [52]. In an in vitro experiment, human colon contents were able to generate 60–250 μM acetaldehyde when incubated with 10–100 mg % of ethanol [142]. The high levels of acetaldehyde attained in the colon likely underlie the correlation between chronic, heavy ethanol consumption and CRC in humans. In alcohol-treated rats, a high concentration of acetaldehyde (50–350 μM) in the colon mucosa has been shown to correlate positively with hyperproliferation of the colon crypt cells [143]. Another evidence for a role of acetaldehyde in CRC initiation emanates from studies showing 3.4 times increase in colon cancer risk among Asians who possess a polymorphism in their ALDH2 enzyme known as ALDH2*2 [144].

As mentioned above, acetaldehyde is metabolized to acetate by ALDH2, ALDH1B1, and ALDH1A1 [145]. The ability of these ALDHs to detoxify acetaldehyde levels is consistent with a role for ALDHs in colon cancer. This hypothesis is supported by the association of ALDH2 deficiency with high incidence of CRC in heavy drinkers [144]. In addition to detoxifying acetaldehyde, ALDH1A enzymes are involved in the formation of RA from retinaldehyde, which plays an important role in cellular proliferation and differentiation [146]. Therefore, RA-generating ALDHs play a critical role in modulating carcinogenesis. In addition, ALDH activity is used as a molecular tool to isolate normal and cancer stem cells of various lineages [147]. Furthermore, the high ALDH expression in cancer stem cells is associated with poor prognosis in CRC [148]. These ALDH bright cells (cells with very high ALDH expression) are more tumorigenic, as reflected by colony-forming capability in vitro and in xenograft-induced tumor formation in vivo [149]. The high expression of ALDH1B1 in both human colon cancers and in animal model of colon polyps has been identified, specifically adenomatous polyposis coli multiple intestinal neoplasia (*Apc* (*Min)/*+) in mice [150]. These mice have the tumor suppressor *Apc* gene mutated, which upregulates oncogenes like *c-Myc* via a dysregulated Wnt signaling [151].

In vivo studies in rats have revealed that retinoids added to the diet reduced colon cancer cell proliferation and prevented azoxymethane-induced aberrant crypt foci (putative precancerous lesions in colon) and colon tumor formation [152, 153].

Alcohol and Upper Aerodigestive Tract Cancer

UADT cancer is among the most frequent cancers in the world. Cancers of the larynx account for approximately 12,000 new cancer cases per year in the United States. Worldwide, approximately 260,000 new cases of oral cancer occur, and more than 125,000 mortalities are attributed to oral cancers each year. Epidemiological studies report an inconsistent relationship between alcohol drinking and UADT cancer mortality; heavy alcohol consumption could increase UADT cancer mortality, especially if combined with tobacco use [154].

For detailed information about alcohol and cancer, the reader is referred to the monographs "*Alcohol and Cancer*" [155] and "*Biological Basis of Alcohol-Induced Cancer*" [156].

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Hepatic and Extrahepatic Malignancies in NAFLD

14

Fabio Nascimbeni and Vlad Ratziu

Introduction

Both hepatic and extrahepatic malignancies are a leading cause of morbidity and mortality among patients with overweight/obesity and/or diabetes mellitus. The increasingly prevalent obesity and related metabolic disorders may cause up to 20 % of cancer deaths and are expected to rapidly overcome the total health burden of cigarette smoking [1–5]. Nonalcoholic fatty liver disease (NAFLD), which is the hepatic manifestation of the metabolic syndrome (MS), has increasingly been associated with an excess occurrence of cancer and increased risk of cancer-related mortality. Natural history studies have consistently reported that malignancies are among the most common causes of mortality, accounting for up to one third of deaths in NAFLD patients with diabetes [6–9]. First and foremost, hepatic neoplasms, in particular hepatocellular carcinoma, have emerged as a notable threat to nonalcoholic steatohepatitis (NASH) patients, sometimes occurring in the absence of cirrhosis [10, 11]. Recent studies suggest that the NAFLD-related oncologic spectrum might extend beyond the liver, to colorectal cancer [12, 13]. In this chapter, we will review the association between NAFLD and hepatic and extrahepatic malignancies.

Malignancy-Related Morbidity and Mortality in NAFLD

Results from epidemiological studies indicate that adiposity contributes to the increased incidence and death from several cancer types, including colon, breast, endometrium, kidney, esophagus, gastric cardia, pancreas, gallbladder, and liver. There are positive linear trends in cancer death rates with increasing body mass index and men and women with a body mass index of at least 40 kg/m^2 have death rates from all cancers that are 52 % and 62 % higher, respectively, than the rates in men and women of normal weight [1, 2]. Similarly, diabetes has a 25 % excess risk of death from cancer, and results are specifically associated with malignancies of the liver, pancreas, colorectum, endometrium, ovary,

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breast, and bladder [4, 5]. Given the close link between obesity-related insulin resistance and NAFLD, it might be anticipated that NAFLD itself is associated with increased cancer burden and significant mortality from malignancy. Indeed, a Danish population-based study found an increased risk of hepatic and extrahepatic malignancies among 1800 subjects discharged from hospital with a diagnosis of NAFLD compared with the general population (standardized incidence ratio: 1.3, 95 % CI: 1.1–1.6). Site-specific risks were significantly higher in NAFLD patients for primary liver cancers (standardized incidence ratio: 4.4, 1.2–11.4) and for pancreas (standardized incidence ratio: 3.0, 1.3–5.8), kidney (standardized incidence ratio: 2.7, 1.1–5.6), and colon malignancies (standardized incidence ratio: 1.6, 0.9–2.9) [12]. Two recent retrospective Japanese studies specifically assessed the development of malignancies in NAFLD. The first study on 1600 ultrasound-diagnosed NAFLD patients aged 60 years or older and followed over a mean of 8.2 years showed that the 10-year cumulative rate of cancer development was 13.9 %. Most common malignancies in NAFLD were gastric (20.4 %), colon (18.6 %), prostate (12.6 %), and lung cancer (10.2 %) in addition to hepatocellular carcinoma (HCC) (6.0 %) [14]. A second study included 312 biopsy-proven NAFLD patients and showed that 1.9 % of patients developed HCC and 6.4 % developed extrahepatic cancers (6.4 %) during a median follow-up period of 4.8 years. Again, the most prevalent malignancies were gastric cancer (20.8 %), followed by HCC (16.7 %), lung cancer (16.7 %), pancreatic cancer (12.5 %), colorectal cancer (8.3 %), and breast cancer (8.3 %). Interestingly, six out of the eight deaths registered during the study were malignancy related [15]. An early study on 132 patients with biopsy-proven NAFLD patients with a mean 8.3-year follow-up has shown that malignancies were the first cause of death, with 11/45 (24 %) of deaths including one from primary liver cancer [6]. A subsequent retrospective community-based cohort study from Olmsted County, USA, among 420 NAFLD patients mainly diagnosed by imaging with a mean follow-up of 7.6 years, confirmed this finding. Extrahepatic malignancies determined 15 out of 53 deaths (28 %) and hepatocellular carcinoma was responsible of death in one additional patient (2 %) [7]. Interestingly, a recent community study from the same US population showed that in patients with diabetes, NAFLD significantly increases malignancy-related deaths: from 18 % when NAFLD was absent to 37 % (9/27 from extrahepatic malignancies and one more case from HCC) when it coexisted with diabetes [9]. A large retrospective community-based study from NHANES III data showed that, after a median follow-up of 8.7 years, cancer mortality (19/80 deaths: 24 %) was second only to cardiovascular mortality among 817 individuals with NAFLD, which was not different from that of a control group of subjects without liver disease (22 %) [8]. This might be explained by the rather narrow definition of NAFLD which was based on increased aminotransferases. Similarly, in a series of 229 biopsy-proven NAFLD patients followed up for a mean of 26.4 years, malignancy was a prominent cause of death: 18 of the 96 deaths were due to non-gastrointestinal malignancy, four to gastrointestinal malignancy, and five to HCC. Only the HCC deaths were significantly higher than those in a control, Swedish population, with a hazard ratio of 6.55 (95 % CI: 2.14–20.03) [16].

In summary, at present, there is convincing evidence that cancer morbidity and mortality in NAFLD patients is not negligible, especially for primary liver cancer. Whether this neoplastic risk is increased by NAFLD itself or simply mirrors the underlying obesity-related insulin resistance condition has not been addressed so far. Also, specific surveillance/screening strategies cannot be recommended as current data is insufficient for evidence-based recommendations.

Hepatic Malignancies in NAFLD

HCC

HCC is the fifth and seventh most common cancer, in men and women, respectively, and the second leading cause of cancer-related mortality

in the world, reflecting the poor prognosis of this disease [17]. The majority of HCC cases occur in less developed countries, principally East Asia and sub-Saharan Africa, typically associated with chronic viral hepatitis B and C. Interestingly, although the incidence of HCC in these countries is decreasing, its incidence in developed countries has been increasing over the last three decades and paralleled the epidemic of obesity and diabetes [18, 19]. For instance, in the USA, the incidence of HCC increased fourfold since the late 1970s, and this cancer became the fastest rising cause of malignancy-related deaths. According to population-based Surveillance, Epidemiology, and End Results (SEER) registry data, the overall HCC age-adjusted incidence rates raised from 1.6 per 100,000 individuals in 1975 to 6 per 100,000 individuals in 2010, with men being at almost three times higher risk than women [10, 20, 21]. The main risk factor for the development of HCC is HBV- and HCV-cirrhosis and alcoholic liver disease. While almost half of the increase in HCC cases was attributed to the aging HCV cohort, up to 30–35 % of HCCs were not associated with either viral hepatitis or alcohol abuse [17, 22, 23]. Despite the low incidence of HCC described in NAFLD patients (who are for the most part non-cirrhotic) as compared to the relative risk in other forms of cirrhosis [24–31], the population attributable fraction (PAF) of NAFLD-HCC is much higher than that of other etiologies because of the prevalence of obesity and diabetes. A population-based study of SEER registry-diagnosed HCC cases reported that the highest PAF was for diabetes/obesity (36.6 %), followed by alcohol-related disease (23.5 %), HCV (22.4 %), and HBV (6.3 %) [21, 32]. In the USA, NAFLD is becoming a leading cause of HCC and the second most common cause of HCC in patients listed for liver transplantation [33]. In England, similar data confirm a higher than tenfold increase in NAFLD-related HCC from 2000 to 2010 [34]. Worryingly, convincing data showed that metabolic risk factors and concurrent NAFLD significantly increase the risk of developing HCC in patients with chronic liver diseases of different etiologies [35–38]. Finally, while HCC almost invariably develops on the ground of cirrhosis in the majority of chronic liver diseases, there is increasing evidence that HCC associated with NAFLD may occur in the absence of advanced fibrosis [34, 39, 40].

HCC in Obesity and Diabetes

The association between either obesity or diabetes and HCC is based on several large-scale epidemiological studies and meta-analyses. A very large prospective study involving more than 900,000 US adults reported a significant positive linear trend in death rate from primary liver cancer with increasing BMI for both men and women; however, this excess risk was higher among men than among women. As compared with men of normal weight, men with a BMI of at least 35 kg/m^2 had a relative risk as high as 4.52 of death for liver cancer, whereas this relative risk was 1.68 in women at the same BMI group [1]. A very recent population-based cohort study of 5.24 million UK adults confirmed that BMI was positively associated with liver cancer development (hazard ratio 1.19, 1.12–1.27 per each 5 kg/m^2 increase in BMI). Again this association was more marked in men than in women; in women, there was a modest linear increase in risk with increasing BMI (1.14), whereas in men, the risk associated with higher BMI was substantial but only above the cutoff of 22 kg/m^2 (1.30). With the assumption of causality, the authors estimated that more than 10 % of liver cancers are attributable to overweight/obesity [41]. A meta-analysis of 11 cohort studies with a total of 6142 cases of liver cancer confirmed the increased risk in overweight/obese individuals; compared with persons of normal weight, overweight and obese subjects had relative risks of liver cancer 17 % and 89 % higher, respectively [42]. A more recent meta-analysis including 21 prospective studies with 17,624 cases of primary liver cancer strengthened these findings describing a summary relative risk of 1.39 for a 5-unit increase in BMI; in addition, the most pronounced increment in the risk was observed at a BMI higher than 32 kg/m^2 and comorbidity with cirrhosis and/or hepatitis further increased the risk [43].

Similar results derive from studies addressing the relationship between diabetes and HCC. An

analysis of individual data from 97 prospective studies in 820,900 people showed that diabetes was associated with an increased risk of death from liver disease and from cancer; in particular, the highest hazard ratio was for death from liver cancer with twofold (2.16) higher risk than in nondiabetics [5]. Other studies from different countries and ethnicities confirmed a twofold excess risk of liver cancer occurrence and/or death in both sexes in diabetic individuals [44–47]. A first meta-analysis including 26 studies confirmed that diabetes was significantly associated with HCC in both case-control and cohort studies with similar risk rates (2.5 compared to individuals without diabetes) [48]. An updated meta-analysis of 25 cohort studies showed a positive association between diabetes and increased risk of both HCC incidence and mortality (summary relative risk of 2.01 and 1.56, respectively); this risk was independent of gender, geographic location, presence of cirrhosis, alcohol consumption, or chronic viral hepatitis [49]. Moreover, as reported for obesity, diabetes acts synergistically with other factors in determining HCC risk [37, 38, 47, 50]. Also, a few studies and two meta-analyses report on a significant association between MS and increased risk of HCC [51–54]. In particular, Turati et al. suggested that the risk of HCC increases with the number of components of the MS [52]. Finally, a large prospective multicentric study of 578,700 European adults with 266 cases of liver cancer showed that BMI, glucose, and MS were all positively associated with risk of primary liver cancer [55].

In summary, in line with biological plausibility, there is strong epidemiological evidence supporting a link between obesity-related insulin resistance, metabolic risk factors, and increased risk of HCC. These findings pave the way for an association between HCC and NAFLD, since NAFLD is the major hepatic manifestation of obesity and related metabolic disorders.

HCC in NASH-Cirrhosis and Cryptogenic Cirrhosis

The first circumstantial evidence of HCC being part of the NAFLD spectrum dates back to 1990, when, in their landmark publication, Powell et al. described a patient with NASH-cirrhosis documented on the initial biopsy who ultimately died from multifocal HCC developed at 5 years of observation [56]. Since then, many case reports and case series and transversal and longitudinal studies have documented the development of HCC on NAFLD, especially on a cirrhotic background (reviewed in [10, 11]).

Longitudinal studies

Several longitudinal studies from Europe, the USA, Australia, and Asia specifically investigated the natural history and the risk of HCC in a cohort of NASH with cirrhosis or advanced fibrosis or cryptogenic cirrhosis presumably NAFLD related on the basis of metabolic risk factors [24–31, 57–61] (Table 14.1). One of them was a population-based cohort study [57], while the others were clinic-based cohort studies; all but one had at least one comparison cirrhosis cohort [59], mainly HCV related. A minority of them had a prospective design [24, 25, 27, 29–31, 59, 60]. In a retrospective nationwide Danish population-based study evaluating HCC risk in cirrhosis, Sorensen et al. reported that 35 of the 2430 cases (1.4 %) with a hospital discharge diagnosis of cryptogenic cirrhosis developed HCC during a follow-up of 5 years or longer; the standardized incidence rate for HCC in patients with cryptogenic cirrhosis was 43/100,000 person-years, lower than that observed in alcohol-related cirrhosis but similar to the rates described in primary biliary cirrhosis and virus-related cirrhosis [57]. Similarly, a retrospective French study showed a comparable hepatocarcinogenic potential between obesity-related cryptogenic cirrhosis and HCV-related cirrhosis (cumulative HCC incidence 29.6 % vs. 21.2 % over a 1.8–2-year follow-up period) [58]. These findings were confirmed by two recent Japanese studies suggesting that advanced NASH/NASH-cirrhosis has similar HCC development rate with respect to HCV-related advanced fibrosis (40 % in both groups over a mean follow-up period of 85.6 months) [60] and alcohol-related cirrhosis (5-year incidence rate 10.5 % vs. 12.3 %) [61], respectively. However, other longitudinal studies reported cumulative HCC incidence or mortality lower in NASH-cirrhosis or cryptogenic

Table 14.1 Characteristics and outcomes of longitudinal studies assessing HCC risk among NASH-cirrhosis/cryptogenic cirrhosis

Author, year [ref.]	Country/setting/period	Study design/cohort definition/control group	Number of patients/follow-up	HCC incidence in cohort vs. control group	HCC mortality in cohort vs. control group
Sorensen et al., 1998 [57]	Denmark/national hospitalizations registry/1977–1989	Population-based, retrospective/ICD nonspecified cirrhosis (ns-C)/ICD alcoholic cirrhosis (AL-C), primary biliary cirrhosis (PBC), chronic hepatitis (CH)	11,605 (2430 ns-C)/5.5 years for men and 5.9 years for women in ns-C	Cumulative incidence: 1.4 % ns-C, 2.0 % AL-C, 1.3 % PBC, 1.1 % CH; standardized incidence ratio Danish population: 43 ns-C, 71 AL-C, 47 PBC, 43 CH	NR
Ratziu et al., 2002 [58]	France/single institution/1988–2000	Clinic-based, retrospective/overweight (O-CC) and lean cryptogenic cirrhosis (L-CC)/HCV-cirrhosis (HCV-C)	27 O-CC, 10 L-CC, 391 HCV-C (85 HCV-C matched with O-CC)/1.8 years in O-CC, 3.6 years in L-CC, 3.4 years in HCV-C (2 years in HCV-C matched with O-CC)	Cumulative incidence: 29.6 % O-CC, 0 % L-CC, 15.6 % HCV-C (21.2 % HCV-C matched with O-CC)	NR
Hui et al., 2003 [24]	Australia/single institution/1985–2002	Clinic-based, prospective/NASH-cirrhosis (biopsy)/untreated and nonresponder HCV-cirrhosis	23 NASH-cirrhosis, 46 age- and sex-matched (23+23) HCV-cirrhosis/7 years in NASH-cirrhosis, 6.7 years in untreated HCV, 6.9 in nonresponder HCV	Cumulative incidence: 0 % NASH-cirrhosis, 21.7 % untreated HCV-cirrhosis, 13.0 % nonresponder HCV-cirrhosis	HCC deaths/all cause deaths: 0/6 in NASH-cirrhosis, 3/10 in untreated HCV, 2/8 in nonresponder HCV
Sanyal et al., 2006 [25]	USA/single institution/1992–2004	Clinic-based, Prospective/NASH-cirrhosis (biopsy)/untreated or nonresponder HCV-cirrhosis	152 NASH-cirrhosis, 150 HCV-cirrhosis matched for age, sex, child class, and year of enrollment/10 years	Cumulative incidence: 6.7 % NASH-cirrhosis, 17.0 % HCV-cirrhosis	HCC deaths/all cause deaths: 2/29 in NASH-cirrhosis, 8/44 in HCV-cirrhosis
Kojima et al., 2006 [26]	Japan/single institution/1990–2004	Clinic-based, retrospective/ cryptogenic cirrhosis (CC)/HCV-cirrhosis, HBV-cirrhosis	24 CC, 48 HCV-cirrhosis, 24 HBV-cirrhosis matched for age, sex, and child class/5.7 years CC, 5.9 years viral (HCV+HBV) cirrhosis	Cumulative incidence: 37.5 % in CC, 73.6 % in viral cirrhosis	HCC deaths/ liver-related deaths: 2/5 in CC, 29/39 in viral cirrhosis
Yatsuji et al., 2009 [29]	Japan/single institution/1990–2006	Clinic-based, prospective/NASH-cirrhosis (biopsy)/HCV-cirrhosis	68 NASH-cirrhosis, 69 sex- and age-matched HCV-cirrhosis/3.4 years in NASH-cirrhosis, 6.2 years in HCV-cirrhosis	5-year incidence rate: 11.3 % in NASH-cirrhosis, 30.5 % in HCV-cirrhosis	HCC deaths/all cause deaths: 9/19 in NASH-cirrhosis, 19/28 in HCV-cirrhosis

(continued)

Table 14.1 (continued)

Author, year [ref.]	Country/setting/period	Study design/cohort definition/control group	Number of patients/follow-up	HCC incidence in cohort vs. control group	HCC mortality in cohort vs. control group
Hashimoto et al., 2009 [59]	Japan/single institution/1990–2007	Clinic-based, prospective/NASH with advanced fibrosis (biopsy)/no control group	137/3.4 years	5-year cumulative incidence: 7.6 %	HCC deaths/all cause deaths: 12/26
Ascha et al., 2010 [30]	USA/single institution/2003–2007	Clinic-based, prospective/NASH-cirrhosis/HCV-cirrhosis	195 NASH-cirrhosis, 315 HCV-cirrhosis/2.7 years in NASH-cirrhosis, 3.4 years in HCV-cirrhosis	Yearly cumulative incidence: 2.6 % NASH-cirrhosis, 4.0 % HCV-cirrhosis	NR
Bhala et al., 2011 [27]	International/4 institutions (USA, UK, Australia, Italy)/1984–2006	Clinic-based, prospective/NAFLD with advanced fibrosis or child A cirrhosis/HCV with advanced fibrosis or child A cirrhosis	247 NAFLD, 264 HCV/7.1 years in NAFLD, 6.2 years in HCV	Cumulative incidence: 2.4 % NAFLD, 6.8 % HCV	HCC deaths/all cause deaths: 3/33 in NAFLD, 12/25 in HCV
O'Leary et al., 2011 [28]	USA/single institution/2002–2008	Clinic-based, retrospective/NASH-cirrhosis or cryptogenic cirrhosis (NASH-CC) listed for OLT/HCV-cirrhosis listed for OLT	217 NASH-CC, 645 HCV-cirrhosis/1.0 year in NASH-CC, 1.0 year in HCV-cirrhosis	Yearly cumulative incidence: 2.7 % NASH-CC, 4.7 % HCV-cirrhosis	NR
Amarapurkar et al., 2013 [31]	India/single institution/2010–2011	Clinic-based, prospective/NASH-cirrhosis and cryptogenic cirrhosis (CC)/HBV-cirrhosis and HCV-cirrhosis	41 NASH-cirrhosis, 104 CC, 111 HBV-cirrhosis, 83 HCV-cirrhosis/6.8 years in NASH-cirrhosis, 5.7 years in CC, 5.9 years in HBV-cirrhosis, 6.1 years in HCV-cirrhosis	Yearly cumulative incidence: 0.46 % NASH-cirrhosis, 0.6 % CC, 1.5 % HBV-cirrhosis, 3.6 % HCV-cirrhosis	NR
Hashizume et al., 2013 [60]	Japan/single institution/2003–2011	Clinic-based, prospective/NASH with advanced fibrosis (biopsy)/HCV with advanced fibrosis	Only female patients: 20 NASH, 20 HCV matched for age and BMI/7.1 years in NASH, 6.8 years in HCV	Cumulative incidence: 40 % NASH, 40 % HCV	HCC deaths/all cause deaths: 2/5 in NASH, 7/10 in HCV
Kodama et al., 2013 [61]	Japan/single institution/1990–2010	Clinic-based, retrospective/NASH-cirrhosis (biopsy)/alcoholic cirrhosis	72 NASH-cirrhosis, 85 alcoholic cirrhosis/4.2 years in NASH-cirrhosis, 3.0 years in alcoholic cirrhosis	5-year incidence rate: 10.5 % NASH-cirrhosis, 12.3 % alcoholic cirrhosis	NR

cirrhosis patients than in HCV-cirrhosis controls [24–31]. Indeed, in these studies, the cumulative incidence of HCC ranges from 0 % over a 7-year follow-up period [24] to 37.5 % during a follow-up of 5.7 years [26] in NASH-cirrhosis/cryptogenic cirrhosis and from 6.8 % over a follow-up of 6.2 years [27] to 75 % during a follow-up of 6.5 years in HCV-related cirrhosis [26]. Accordingly, yearly cumulative incidence of HCC ranges from 0–0.05 % [24, 27] to 2–3 % [28–30] in NASH-cirrhosis/cryptogenic cirrhosis and from 0.15 % [27] to 4–5 % [28–30] in HCV-related cirrhosis, respectively.

In summary, there is strong evidence to support the risk of HCC in patients with NASH and advanced fibrosis or cirrhosis. However, given the generally small sample size, the modest follow-up period, and the heterogeneity in study design, in cohort definition and severity, and in primary data sources, it is not possible to clearly define the actual size of this risk.

Transversal (case-control and cross-sectional) studies

There are numerous transversal studies from different countries and ethnicities comparing cases with NASH-cirrhosis or cryptogenic cirrhosis-related HCC with at least one HCC control group. A milestone case-control Italian study, dating back to 2002, demonstrated that features suggestive of the MS, including obesity, type 2 diabetes, insulin resistance, and dyslipidemia, are observed more frequently in patients with HCC complicating cirrhosis of unknown etiology than in age- and sex-matched patients with HCC due to alcoholic or viral cirrhosis [62]. In line with those findings, another study from the USA confirmed that a large majority of patients with HCC and cryptogenic cirrhosis had a prior histological diagnosis of NASH or clinical features associated with NAFLD [63]. These seminal studies suggested that the presence of metabolic disorders may first lead to fatty liver and ultimately to HCC through NASH, fibrosis, and cirrhosis. Subsequent case-control and cross-sectional studies found that HCC attributed to NASH-cirrhosis or cryptogenic cirrhosis occurred at an older age and was more often associated with features of the MS, especially diabetes and overweight/obesity, than control groups with HCC complicating other chronic liver diseases (reviewed in [11]). These findings strongly suggest that HCC is a late complication of NASH.

Case reports and case series

Only two cases of HCC in patients with NASH-cirrhosis had been reported in longitudinal studies until 1999 [6, 56]. Thereafter, several observations of HCC in NASH-cirrhosis have been published, mostly from Japan (reviewed in [10, 11]). The first two case reports, one from Brazil and one from Japan, described a male and a female patient aged 62 and 72 years, both with type 2 diabetes and known, histologically confirmed NASH, who developed HCC on a cirrhotic liver, 4 and 10 years, respectively, after the diagnosis of NASH [64, 65]. In a single-center case series of 82 Japanese patients with NASH, 6 patients presented with or developed HCC, all of them with NASH-cirrhosis [66]. These and subsequent case reports/case series confirmed that HCC is not an unusual event in NASH patients, especially those reaching the cirrhotic stage.

HCC in Non-cirrhotic NASH (Simple Steatosis and Steatohepatitis Without Advanced Fibrosis)

Many reports now suggest that NAFLD-related HCC may also occur in patients with NASH but without advanced fibrosis and even in simple steatosis.

Longitudinal studies

Clinic-based, longitudinal studies assessing the natural history of NAFLD patients, not restricted to those with cirrhosis or advanced fibrosis, showed HCC incidence and mortality rates ranging from 0 to 6 % during follow-up periods of 1–2 decades [6–8, 14–16, 60, 67–73] (Table 14.2). In a prospective US population-based study analyzing the NHANES cohort, none of 817 NAFLD patients, identified on the basis of elevated liver enzymes without other causes of liver disease, developed HCC during a median follow-up of 8.7 years [8]. Similarly, a Danish cohort study with a follow-up longer than 20 years

Table 14.2 Characteristics and outcomes of longitudinal studies assessing HCC risk among NAFLD patients, not restricted to those with cirrhosis or advanced fibrosis

Author, year [ref.]	Country/setting/period	Study design/cohort definition	Number of patients/ cirrhosis prevalence/ follow-up	HCC cases in the whole cohort/HCC cases in pts without advanced fibrosis-cirrhosis	HCC mortality in the whole cohort/HCC mortality in pts without advanced fibrosis-cirrhosis
Matteoni et al., 1999 [6]	USA/single institution/1979–1987	Clinic-based, retrospective/ NAFLD (liver biopsy)	132 patients/15 % with cirrhosis/8 years	NR	HCC deaths/all cause deaths: 1/48
Adams et al., 2005 [7]	USA/resources of Rochester Epidemiology Project/1980–2003	Population-based, prospective/ NAFLD (imaging or liver biopsy) and cryptogenic cirrhosis with prior metabolic syndrome	435 patients/5 % with cirrhosis (2 % at diagnosis and 3 % during follow-up)/7.6 years	2 cases of HCC, all in patients with cirrhosis	HCC deaths/all cause deaths: 1/53
Ekstedt et al., 2006 [67]	Sweden/single institution/1988–1993	Clinic-based, retrospective/ NAFLD (liver biopsy for persistently elevated liver enzymes)	129 patients/3.1 % with cirrhosis at baseline/13.7 years	3 cases of HCC, all in patients with cirrhosis (1 cirrhosis at baseline and 2 cirrhosis developed during follow-up)	HCC deaths/all cause deaths: 1/25
Ong et al., 2008 [8]	USA/NHANES III/1988–2000	Population-based, prospective/ NAFLD (elevated aminotransferases in the absence of other chronic liver diseases)	817 patients/cirrhosis NR/8.7 years	NR	HCC deaths/all cause deaths: 0/80
Dam-Larsen et al., 2009 [68]	Denmark/single institution/1976–2004	Clinic-based, retrospective/ NAFLD without inflammation or significant fibrosis (liver biopsy)	170 patients/0 % with cirrhosis/20.4 years	NR	HCC deaths: 0
Rafiq et al., 2009 [69]	USA/two institutions/NR	Clinic-based, retrospective/ NAFLD (liver biopsy)	173 patients (72 NASH, 101 non-NASH)/NR/10.5 years in NASH, 13.0 years in non-NASH	NR	HCC deaths/all cause deaths: 1/78
Sanyal et al., 2010 [70]	USA/National Insurance database/2002–2008	Population-based, retrospective/ NAFLD (ICD codes)	18 million persons, 4 % with ICD codes for NAFLD/NR	Cumulative incidence: 0.3 % HCC with NAFLD (only 46 % of HCC-NAFLD cases had ICD codes for cirrhosis)	NR

Soderberg et al., 2010 [71]	Sweden/single institution/1980–2008	Clinic-based, retrospective/ NAFLD (liver biopsy for persistently elevated liver enzymes)	118 patients/7.6 % with cirrhosis at baseline/21 years	NR	HCC deaths/all cause deaths: 5/47 (3 patients had cirrhosis at baseline, 1 had stage 3 fibrosis and 1 stage 1 fibrosis at baseline)
Arase et al., 2012 [14]	Japan/single institution/1994–2007	Clinic-based, retrospective/ NAFLD (ultrasonography)	1600 patients/NR/8.2 years	10 HCC cases, corresponding to development rate of 0.78/1000 person-years (0.83/1000 person-years in male, 0.63/1000 person-years in female)	NR
Kawamura et al., 2012 [72]	Japan/single institution/1997–2010	Clinic-based, retrospective/ NAFLD (ultrasonography)	6508 patients/NR/5.6 years	16 HCC cases, corresponding to annual rate of 0.043 %	NR
Hashizume et al., 2013 [60]	Japan/single institution/2003–2011	Clinic-based, prospective/NASH with mild fibrosis (liver biopsy)	19 female patients/0 % with cirrhosis/7.6 years	Cumulative incidence: 0 %	HCC deaths/all cause deaths: 0/1
Ekstedt et al., 2014 [16]	Sweden/two institutions/1980–1993	Clinic-based, retrospective/ NAFLD (liver biopsy for persistently elevated liver enzymes)	229 patients/11.8 % advanced fibrosis or cirrhosis at baseline/26.4 years	NR	HCC deaths/all cause deaths: 5/96 (1 patient had fibrosis stage F0 and 2 patients had fibrosis stage F2 at baseline; however all developed cirrhosis during follow-up)
Onnerhag et al., 2014 [73]	Sweden/Malmo Preventive Project/1974–1992	Population-based, prospective/ NAFLD (liver biopsy)	36 patients/25 % with cirrhosis/27.0 years	5 HCC cases, corresponding to cumulative incidence of 13.9 %; all cases occurred in patients with cirrhosis	HCC deaths/all cause deaths: 5/21
Seko et al., 2014 [15]	Japan/single institution/1999–2013	Clinic-based, retrospective/ NAFLD (liver biopsy)	312 patients (176 NASH, 136 non-NASH)/8.5 % with cirrhosis among NASH/4.8 years	6 HCC cases, corresponding to annual incidence of 0.4 %. All cases occurred in NASH patients. 1 patient had fibrosis stage 1 and 1 fibrosis stage 2 at baseline	HCC deaths/all cause deaths: 1/8

found no HCC in any of the 170 subjects with biopsy-proven NAFLD and no significant fibrosis at baseline [68]. Conversely, a retrospective Japanese study reported a cumulative HCC incidence of 5.3 % at 10 years in biopsy-proven NAFLD patients; interestingly, all six patients who developed HCC had NASH, and two of them had fibrosis stages 1 and 2 at baseline [15]. Also a Swedish cohort study reported cumulative HCC mortality rates of 3 % in biopsy-proven NAFLD and 6 % in NASH patients during a 21-year follow-up; notably, one out of five HCC deaths occurred in a patient with baseline stage 1 fibrosis [71]. Recently Ekstedt et al. confirmed HCC as a cause of death in NAFLD patients. Indeed, they registered 5 HCC-related deaths among 229 biopsy-proven NAFLD patients followed up for 33 years; 3 HCC cases were observed in patients with NASH without advanced fibrosis at baseline; however, all of them had developed cirrhosis during follow-up [16]. A large retrospective study enrolling more than 6000 Japanese patients with ultrasound-diagnosed NAFLD reported 16 (0.25 %) new HCC cases during a 5.6-year follow-up period; patients older than 60 years, with diabetes, increased AST levels, and thrombocytopenia, were at increased risk of HCC [72]. Interestingly, a recent retrospective US population-based study analyzing a national health insurance database covering 18 million lives yearly from 2002 to 2008 found that NAFLD without other chronic liver diseases was the leading cause of HCC, accounting for 38.2 % of HCC cases, while cirrhosis was reported in only 46 % of these patients (by ICD diagnoses) [70]. These findings may suggest that at least in a subset of NAFLD patients, HCC may occur in the absence of advanced fibrosis.

Transversal (case-control and cross-sectional) studies

In case-control and cross-sectional studies not restricted to NASH-cirrhosis-related HCC, cirrhosis accounts for the majority albeit not all HCC cases associated with NAFLD. In a Japanese cohort of 34 NASH patients with HCC, Hashimoto et al. found that 12 % of patients presented F1-2 stages of fibrosis [59]. In another Japanese study, Abe et al. described that one out of ten NAFLD-related HCC and four out of seven cryptogenic HCC did not have cirrhosis; moreover, patients with nonviral HCC had a higher rate of early-stage cirrhosis than those with viral HCC [74]. Recently, in a European and US population, 47 % of patients with NAFLD-HCC and 61 % of patients with cryptogenic HCC had no evidence of cirrhosis; this was in striking contrast with the 93–95 % prevalence of cirrhosis in patients with viral or alcohol-related HCC [75]. In a UK population, 23 % of NAFLD-HCC patients had no clinical, radiological, or histological evidence of cirrhosis vs. 0 % and 3.1 % of HCC cases due to alcohol or HCV, respectively [34]. This was confirmed in an American series where only 73 % of NASH HCC had bridging fibrosis or cirrhosis vs. 94 % for HCV- and/or alcohol-related HCC [76]. Finally, Paradis et al. studied 31 patients with HCC and features of the MS as the only risk factor for liver disease. Early fibrosis (F0–F2) was more common than in HCC patients with overt causes of chronic liver disease (65 % vs.26 %). Intriguingly, HCCs that developed in non-fibrotic livers were more often well differentiated despite their larger size and derived from the malignant transformation of a preexisting hepatocellular adenoma in a substantial proportion of cases (5/20) [39].

In summary, NAFLD-related HCC may arise in the absence of significant liver fibrosis, suggesting that liver carcinogenesis related to NAFLD may be more complex than the usual multistep process fibrosis-cirrhosis-HCC.

Case reports and case series

A recent review reported that, between 2004 and 2011, at least 116 cases of HCC have been described in histologically confirmed NAFLD without cirrhosis [10]. Arguably some of these might have been labeled F3 because of sampling error or incomplete cirrhosis. But many reports of HCC in NAFLD without significant fibrosis have since been published, suggesting that non-cirrhotic HCC may be more common in NAFLD than in other chronic liver diseases [34, 76–84]. For instance, in a French surgical series of HCC,

24 % had no or minimal fibrosis in the non-tumoral liver and more than half of these had changes consistent with NAFLD/NASH (hepatocyte steatosis, hepatocyte necrosis, and inflammation) [85]. The largest series of non-cirrhotic NAFLD-related HCC was recently described by Yasui et al. in 87 Japanese patients with NASH and HCC; a significant proportion were stage 1 [10 (11 %)] or 2 [15 (17 %)]. Male gender, obesity, diabetes, and features of the MS predominated in this series. Of note, men developed HCC at a less advanced stage of fibrosis than women, and the prevalence of cirrhosis was significantly lower among males compared to females (39 % vs. 70 %) [40]. NAFLD-related HCC not only has been reported in patients with steatohepatitis and different stages of fibrosis but also in patients with stigmata of MS and histological evidence of NASH without fibrosis and even in patients with steatosis without fibrosis or necroinflammation. Bullock et al. described two male patients aged 74 and 64 years, both with full-blown MS who presented with HCC and histological evidence of moderate macro- and microvesicular steatosis with mild lobular inflammation and absence of fibrosis in the non-tumoral liver parenchyma [86]. Guzman et al. described three cases of HCC, two females and one male, aged from 45 to 70 years, with features of MS and histopathologic evidence of bland steatosis, without hepatocellular ballooning, lobular inflammation, and fibrosis [87]. Recently, another case of HCC on simple steatosis without inflammation and fibrosis has been described in a 72-year-old obese Japanese man [88]. Surprisingly and somehow alarmingly, these findings have been also confirmed in children [81].

In summary, the whole histological spectrum of NAFLD can lead to HCC. This mandates the identification of risk factors of HCC development in NAFLD: apart from advanced fibrosis and cirrhosis, current data only point to male gender, age, and features of MS. Equally critical is to understand the underlying mechanisms of hepatocarcinogenesis in insulin resistance. Hopefully this will lead to monitoring strategies in at-risk patients but current evidence-based data are lacking except for patients with NASH-cirrhosis.

Hepatocellular Adenoma

Over the last decade, the pathogenic paradigm of hepatocellular adenomas (HCAs), benign tumors occurring predominantly in young women, has evolved from a relatively rare disease, typically associated with long-term first-generation oral contraceptives use [89, 90], to a more common condition occurring in patients with overweight/obesity and related dysmetabolic comorbidities, as a result of ongoing hyperinsulinism, proinflammatory state, and sex hormone unbalance (Table 14.3) [91–98]. Of particular concern, obesity and MS have been linked with tumor growth and malignant transformation of HCAs [91–93]. The first description of an association between HCAs and MS-related fatty liver dates back to 2005 when Brunt and colleagues documented a case of hepatic adenomatosis arising in a non-cirrhotic liver with NASH [99]. Since then, several case series have been published assuming a relationship between metabolic steatopathy and HCAs, particularly inflammatory HCAs, and arguing that these liver tumors should be added to the list of neoplasms associated with overweight/obesity and features of MS. Paradis et al. in their surgical series of 32 telangiectatic/inflammatory HCAs from 27 patients reported for the first time an association with overweight/obesity. Moreover, significant steatosis outside tumors was observed in 69 % of patients and moderate/severe steatosis in more than 30 % [100]. Accordingly, a recent histomorphological investigation of the non-tumorous liver of 32 resected inflammatory HCAs confirmed that steatosis was very common; it was present in adjacent, non-tumoral liver in 59–70 % of cases. Steatohepatitis was only present in 2/32 patients [101]. Recent case reports described patients with inflammatory adenomas within the entire histological spectrum of NAFLD [102–104]. Comparing 24 patients with radiologically and pathologically proven HCAs with age- and sex-matched controls with other benign liver lesions (hemangiomas), Furlan et al. showed that HCAs occurred more frequently and more often were multiple in patients with hepatic steatosis [105]. Interestingly, an American series of 60 patients with HCAs not

Table 14.3 Review of the literature regarding the association between metabolic syndrome, NAFLD and HCAs

Author, year [ref.]	Methods	Main findings
Brunt et al., 2005 [99]	Case report	Hepatic adenomatosis arising in a non-cirrhotic liver with NASH
Wan der Windt et al., 2006 [96]	Case series of 48 patients with HCAs	22 patients (46 %) had liver steatosis. Liver steatosis was significantly more common in patients with multiple HCAs (59 % vs. 19 %, $p=0.008$)
Bioulac-Sage et al., 2007 [94]	Genotype–phenotype correlation in a series of 93 HCAs	High BMI, alcohol intake, and elevated GGT were significantly associated with inflammatory HCA
Paradis et al., 2007 [100]	Review of a surgical series of 32 inflammatory HCAs from 27 patients	Association of telangiectatic/inflammatory HCAs with overweight/obesity: 17 patients (63 %) had a BMI ≥ 25 kg/m^2, of whom 9 were obese. Significant steatosis outside tumors was observed in 69 % of patients and moderate and severe steatosis in more than 30 %. 9 patients had at least 1 other inflammatory HCA. Foci of well-differentiated HCC in 1 case
Furlan et al., 2008 [105]	Case-control study of 24 patients with radiologically and pathologically proven HCAs compared with age- and sex-matched controls with other benign liver lesions (hemangiomas)	Hepatic steatosis was present in 14/24 cases (58 %) vs. 7/24 controls (29 %) ($p=0.042$). Steatosis was more common in patients with multiple HCAs (9/11, 82 %) than in those with a single HCA (5/13, 38 %) ($p=0.047$). BMI (mean ± SD): 30.1 ± 7.24 kg/m^2 vs. 28.1 ± 5.28 kg/m^2 in cases and controls, respectively ($p=0.456$). 8 cases (33 %) and 2 controls (8 %) had diabetes ($p=0.033$)
Lim et al., 2008 [102]	Case report	Multiple inflammatory HCAs in a background of NASH
Bioulac-Sage et al., 2009 [95]	Genotype–phenotype correlation in a surgical series of 128 HCAs	Inflammatory HCAs were characterized by a BMI > 25 kg/m^2 in 43 % and by steatosis in the non-tumoral liver in 38 % of the cases. Patients with inflammatory HCAs were more frequently exposed to alcohol (22 %)
Paradis et al., 2009 [39]	Analysis of the pathological characteristics of HCC and non-tumoral liver in 31 patients with MS as the only predisposing condition for liver disease	MS-associated HCCs mainly occurred in the absence of significant fibrosis in the background liver (64.5 % fibrosis F0–F2), were more often well differentiated (64.5 %) despite their larger size (8.8 ± 6 cm), and, at least some of them, arose through malignant transformation of a preexisting HCA (5 cases, 16 %), particularly the inflammatory variant (3/5, 60 %). All HCC that developed from a preexisting HCA were observed in the group of patients without significant fibrosis
Watkins et al., 2009 [103]	Case report	Inflammatory HCA associated with hepatic adenomatosis arising within advanced-stage NAFLD (pre-cirrhotic)
Bunchorntavakul et al., 2011 [91]	Survey of 60 patients with HCAs	Overweight and obesity were present in 18 and 55 % of patients. Fatty liver, hypertension, diabetes, and dyslipidemia were reported in 57 %, 42 %, 30 %, and 23 % of patients, respectively. 2 patients had PCOS. 72 % of patients had multiple adenomas. Obesity was more often associated with fatty liver ($p=0.006$), diabetes ($p=0.003$), hypertension ($p=0.006$), dyslipidemia ($p=0.03$), and multiple (85 % vs. 48 %, $p=0.005$) and bilobar (67 % vs. 33 %, $p=0.01$) HCAs. The rate of complete resection of HCAs was significantly lower in obese patients (8 % vs. 69 %, $p=0.004$). In the 26 patients without intervention, tumor size progression was more frequently observed in obese patients (33 % vs. 0 %, $p=0.05$). 3/15 obese patients (20 %) lost ≥5 % body weight and there was no progression in the liver lesions

(continued)

Table 14.3 (continued)

Author, year [ref.]	Methods	Main findings
Farges et al., 2011 [92]	Series of 218 histology proven HCAs screened to identify malignant transformation	Areas of HCC within HCA were observed in 23 patients and the risk of malignant transformation was 4 % in women and 47 % in men. MS was the most frequent condition associated with the markedly increased number of HCC within HCAs in men
Sasaki et al., 2011 [98]	Genotype–phenotype correlation in a series of 14 HCAs	4 patients with inflammatory HCA (57 %) were alcohol drinkers, 2 were overweight/obese (29 %), and 2 had diabetes (29 %). Hepatic fibrosis or cirrhosis in the background liver was seen in 5 and inflammatory HCA (71 %) and steatosis in 4 (57 %)
Van Aalten et al., 2011 [97]	Genotype–phenotype correlation in a surgical series of 71 HCAs in 58 patients	Median BMI 26.6 kg/m^2, 55.6 % of patients was overweight/obese. 38 % had steatosis on non-tumoral liver tissue (33 % grade 1 or 2, 5 % grade 3). Patients with inflammatory HCA were more obese (median BMI 28.7 kg/m^2)
Bioulac-Sage et al., 2012 [93]	Analysis of a large cohort of HCAs	The number of resected HCAs increased faster in the 2001–2011 period (110 patients) compared to the 1990–2000 period (35 patients). This phenomenon concurred with an increasing number of patients overweight or obese (38.1 % vs. 14.2 %). Females still represented the great majority of overweight/obese patients presenting HCA; however, overweight/obese male patients constituted a new entity in the inflammatory HCA and beta-catenin-activated inflammatory HCA subgroups. MS was found only in inflammatory HCA. Steatosis in the non-tumoral liver was found mainly in cases with inflammatory HCA and unclassified HCA. Obesity influenced the decision to treat HCA without surgery
Han et al., 2012 [101]	Histomorphological investigation of the non-tumorous liver of 32 resected inflammatory HCAs	Steatosis was present in 59–70 % of the distant and adjacent non-lesional samples, in accordance with a median BMI of 32 kg/m^2. NASH was present only in 2/32 patients
Liu et al., 2014 [106]	Clinicopathological analysis of 74 non-cirrhotic HCCs from 72 patients	Men were more commonly affected (59 %); however, in the <50-year-old group, women predominated (8:1). Median age was 64 years. 25 % had diabetes, 69 % were overweight/obese, and 58 % had metabolic syndrome. 30 % of non-cirrhotic HCCs had some clinical, morphological, or immunophenotypical associations currently described in HCAs
Nascimbeni et al., 2014 [104]	Case report	Multiple inflammatory HCAs in a background of NASH in a middle-aged woman with full-blown MS and PCOS

only confirmed a high prevalence of overweight/obesity (18/55 %) but also demonstrated that tumor progression was more frequent in obese patients [91]. In addition to being involved in size progression of HCAs, obesity and the MS may also favor malignant transformation of HCAs. In their large series of HCAs with malignant changes, Farges et al. noticed an association between the MS and increasing numbers of HCCs developed with the MS [92]. In support of the hypothesis that HCAs are the likely predisposing condition for MS-associated HCC, a seminal French study demonstrated that HCC in patients with features of MS could be considered a distinct biological entity in terms of both pathogenesis and evolution. As opposed to cirrhosis-related HCC, MS-associated HCCs mainly occurred in the absence of significant

fibrosis in the background liver, were more often well differentiated despite their larger size, and at least some of them arose through malignant transformation of a preexisting liver cell adenoma, particularly the inflammatory variant [39]. A recent independent study confirmed that a substantial fraction (nearly a third) of non-cirrhotic HCC have some clinical, morphological, or immunophenotypical features currently described in HCAs [106].

In summary, HCA occurrence and progression seem to be strongly associated with features of MS. Data suggest that HCA might be the missing link between NAFLD and HCC.

Intrahepatic Cholangiocarcinoma

Cholangiocarcinoma is the second most common primary liver cancer, and the number of cases of intrahepatic cholangiocarcinoma (IH-CCA) has been steadily increasing worldwide. For example, data from the SEER program indicated an increase in the age-adjusted annual incidence of IH-CCA in the USA from 0.13 to 0.58 per 100,000 over a 25-year period, with similar trends observed for many other countries worldwide [107, 108]. Since well-established risk factors of IH-CCA such as biliary tract inflammation and parasitic liver flukes are very infrequent in Western countries, the conditions implicated in the increasing occurrence of IH-CCA remain unclear. Recently, several studies have suggested that obesity, diabetes, features of MS, and possibly NAFLD may contribute to an increased risk of this cancer. Michelini et al. reported on three patients with IH-CCA sharing insulin resistance as underlying risk factor: two with type 2 diabetes and one with obesity and dyslipidemic with NAFLD [109]. A Japanese report described a case of IH-CCA in a 50-year-old man with full-blown MS and histologically proven NASH with pericellular and perivenular fibrosis [110]. Larger case-control studies from geographically diverse regions subsequently confirmed that features of the MS are significantly associated with an increased risk of IH-CCA [51, 55, 108, 111–117]. Obesity was found to be associated with a 56 % higher risk of IH-CCA (95 % CI 1.26–1.94) in a recent meta-analysis including three studies (two from the USA and one from Denmark) with a total study population of 304,134 patients. In the same meta-analysis, data from nine case-control studies (four from the USA, two from China, and one from each of Denmark, Japan, and Korea) with a total study population of 400,617 patients showed that type 2 diabetes mellitus was associated with a 89 % higher risk of IH-CCA (95 % CI 1.74–2.07) [108]. The latter finding has been more recently strengthened by a large American study involving 620 IH-CCA cases showing that diabetes independently increases the risk of IH-CCA three-to fourfold [117]. Moreover, a US analysis of SEER-Medicare data from 743 patients older than 65 years of age with histologically confirmed IH-CCA demonstrated that MS confers an independent and statistically significant 1.6-fold increased risk of IH-CCA [51]. Altogether these findings imply that NAFLD may account for a relevant proportion of idiopathic IH-CCA, as it is the case for cryptogenic HCC. A very recent international multi-institutional study described that among 181 patients with resectable IH-CCA, 31 (17 %) presented definite or borderline NASH in the non-tumoral liver parenchyma [118]. An independent French study has investigated the clinical characteristic and histopathologic changes in the distant non-tumoral liver in 57 patients with peripheral IH-CCA occurring in the absence of cirrhosis or chronic bile duct disease. The authors showed that overweight, diabetes, and MS were common findings (in 67 %, 39 %, and 39 % of patients, respectively) and macrovesicular steatosis in more than 10 % of hepatocytes, present in 67 % of cases, was consensually the main histopathologic change observed in non-tumoral parenchyma, including 11 patients (19 %) with steatohepatitis [119].

In summary, even if a clear causality has not been demonstrated, mounting epidemiological data indicate that metabolic derangements and NAFLD are involved in the development of both types of primary liver cancer, HCC, and IH-CCA.

Extrahepatic Malignancies in NAFLD

Emerging data suggest a link between NAFLD and some extrahepatic cancers typically those closely associated with obesity and diabetes [12–15, 120–123]. In particular, colorectal adenoma and cancer, the third commonest malignancy worldwide, whose risk is clearly increased in subjects with obesity and related metabolic disorders, have been associated with NAFLD by several retrospective studies from Asia, the USA, and Europe [13, 124–129]. Hwang et al. provided the first evidence that such an association might exist. In this Korean community-based cross-sectional study of 2917 subjects undergoing routine colonoscopy, ultrasound-diagnosed NAFLD was significantly and independently associated with a 28 % higher risk of colorectal adenomatous polyps; this risk was even higher for multiple adenomatous polyps [124]. Another study from Austria that included 1211 subjects undergoing screening colonoscopy confirmed that patients with ultrasound-diagnosed NAFLD had a significantly higher prevalence of colorectal lesions than subjects without NAFLD (34 % vs. 21.7 %, $p<0.001$). In particular, NAFLD was independently associated with colorectal adenomas (adjusted OR 1.47) and men exhibited an increased risk of colorectal carcinomas [126]. A recent cross-sectional study from China further confirmed that NAFLD, as diagnosed by ultrasound, is an independent risk factor for colorectal malignant neoplasms detected at screening colonoscopy (adjusted OR 1.87, 95 % CI 1.36–2.57) [129]. Wong et al. were the first to assess the relationship between colorectal neoplasms and NAFLD/NASH defined through proton magnetic resonance spectroscopy and liver biopsy. This cross-sectional study found that NAFLD patients had a higher prevalence of colorectal adenomas (34.7 % vs. 21.5 %) and advanced neoplasms (18.6 % vs. 5.5 %) than non-NAFLD controls. Intriguingly, subjects with NASH had a significantly and independently higher risk for both adenomas (adjusted OR 4.89, 95 % CI 2.04–11.70) and advanced neoplasms (adjusted OR 5.34, 95 % CI 1.92–14.84) than those with simple steatosis, whereas simple steatosis did not confer an increased risk with respect to control subjects [13]. In contrast, a small US study was not able to detect any significant difference in the prevalence of colorectal neoplasms between biopsy-proven NAFLD subjects and controls without NAFLD on sonographic imaging; however, non-NAFLD subjects appeared to have a lower number of adenomas per person [125]. A major limitation of studies with a cross-sectional design is the difficulty in inferring causality; however, two longitudinal studies suggested that a causal relationship between NAFLD and colorectal neoplasms might exist. A retrospective longitudinal cohort study including 5517 Korean women showed that, during a 7-year follow-up period, ultrasound-determined NAFLD had an independently twofold higher risk of adenomatous polyps and a threefold increased occurrence of colorectal cancer, as detected through medical certificate codes for insurance claims [127]. Finally, in a recent retrospective cohort study, Huang et al. reported on 1522 individuals undergoing two consecutive colonoscopies during a 2.4-year interval. Ultrasound-detected NAFLD resulted an independent risk factor for the development of colorectal adenoma after a negative baseline colonoscopy (adjusted OR 1.45, 95 % CI 1.07–1.98) [128]. In summary, available data consistently suggest an association between NAFLD and colorectal neoplasms; however, well-designated, prospective studies with long-term follow-up are needed before a true causal relationship can be confirmed [121].

There are also isolated reports of an increased risk of pancreas, stomach, kidney, prostate, lung, and breast cancers in NAFLD patients [12, 14, 15, 123]. However, these data are preliminary, and their validity remains to be evaluated prospectively.

Conclusion

NAFLD might be associated with an oncologic burden and might be directly involved in the development of primary liver tumors and several types of extrahepatic cancers. The best data

concerns the risk of HCC, which clearly seems to be increased in NAFLD patients, especially when advanced fibrosis or cirrhosis has developed. NAFLD-associated HCC may also arise on NASH without advanced fibrosis or even on simple steatosis. A high priority for future research will be to understand the real magnitude of the association between non-cirrhotic NAFLD and liver cancer. It is already clear that the interplay between NAFLD and primary liver tumors is more complex than the classic linear equation fibrosis-cirrhosis-dysplasia and probably hepatocellular adenoma may be involved in this process. The intriguing links between NAFLD and intrahepatic cholangiocarcinoma and colorectal neoplasms need to be confirmed prospectively. The implementation of individual case-based screening and surveillance programs and the demonstration of their cost efficacy is an unmet clinical need [10, 104, 130].

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15 Treatment of Alcoholic Liver Disease Including Emerging Therapies, Novel Targets, and Liver Transplantation

Jan Petrasek and Gyongyi Szabo

Introduction

Alcoholic liver disease represents a significant disease and financial burden for most societies. While only about 10–15 % of people who drink heavily for extended period of time develop liver cirrhosis and/or severe acute alcoholic hepatitis, these conditions are major causes of liver-related mortality [1, 2]. Published data show that several agents are beneficial in short-term treatment of severe acute alcoholic hepatitis, but none have proven to be effective in all patients when used as monotherapy and most have little or no impact on 6-month mortality [3]. Acute alcoholic hepatitis is a severe condition with massive activation of the inflammatory cascade in an otherwise immunocompromised patient due to the effects of alcohol on the immune system [4].

The pathomechanism of alcoholic hepatitis involves several major elements including alcohol-mediated hepatocyte damage and gut-derived and sterile danger signals leading to the activation of the pro-inflammatory cytokine cascade and increased production of reactive oxygen species [5]. It seems likely that combining therapeutic agents in ways that maximize benefits by targeting different pathways while avoiding the risks of infection and hepatorenal syndrome may be necessary to improve mortality particularly in the most severely ill patients with alcoholic hepatitis. Despite increases in knowledge of mechanisms for AH, mortality is not improving but actually worsening. A recent evaluation of outcome in 661 placebo-treated patients from 19 trials found a mortality rate of 20 % at 30 days and 34 % at a median of 160 days follow-up [6]. These statistics illustrate the grim outcome of acute alcoholic hepatitis despite major advances in critical care and other medical interventions over the last three decades. In this chapter, we describe current standard of care therapies and discuss new therapeutic consideration based on existing preclinical evidence.

Traditional and Current Treatment of Alcoholic Liver Disease and Alcoholic Hepatitis

Abstinence

Cessation of alcohol intake improves the survival of patients with all stages of ALD [7, 8]. In severe AH, it would be expected that the near-death experience would prompt patients to stop abusing alcohol but many patients, most likely as a result of alcohol addiction, return to their prior

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drinking patterns, with prevalence of recidivism between 30 % [9] and 70 % [10]. Lifelong sobriety has been considered vital for recovery from severe AH [3, 11, 12]. Although seemingly intuitive, published data regarding impact of recidivism on survival of patients with severe AH are conflicting, most likely due to inadequate follow-up, as well as inclusion of patients with the entire ALD spectrum [9, 13, 14].

The survival benefit of abstinence becomes apparent when follow-up is sufficiently long, likely more than one year [10, 13], but may be limited only to patients with underlying stage A or B cirrhosis, but not to patients with stage C cirrhosis [13]. In the long-term follow-up, recidivism of ethanol consumption determines mortality to the similar extent as gastrointestinal bleeding [10, 13]. Available data show that abstinence from excessive drinking is the most important predictor of survival in patients with cirrhosis [3, 13–15]. The 3-year survival rate is up to 80 % among patients who abstain from alcohol, as compared with only 20 % in individuals who continue excessive drinking [14].

Abstinence from alcohol does not seem to provide survival benefit in short-term time frame (3–12 months) following severe AH [9, 14]. In this scenario, the major causes of death are acute liver failure, hepatorenal syndrome, and sepsis [9, 10], all of which are primarily attributable to the sustained effect of synthetic liver failure and immune paralysis [16], rather than to the direct effect of ethanol [17]. The vast majority (>90 %) of patients with severe AH have underlying cirrhosis [18, 19]. If they recover from severe AH, the relatively narrow time window between early, recidivism-independent mortality and late, recidivism-dependent mortality should be utilized to focus on follow-up care and on achieving abstinence.

The goal of intervention should be sustained complete abstinence, which is associated with reduction of portal pressure, inflammation, and slower progression to cirrhosis [3, 12]. Establishing abstinence is best achieved by close collaboration between hepatologists and addiction psychiatrists, with support from trained counselors. So-called brief behavioral interventions can be implemented by nonpsychiatric staff and involve educating the patients about the nature of their diagnosis and implementing a behavioral change. This intervention is best suited for patients who experience risk drinking without dependence. Brief behavioral interventions in an outpatient setting have significantly increased the chances of heavy drinkers to reduce drinking at 6 and 12 months [20–22]. Up to one-third of patients remain abstinent for one year after a single course of intervention by a substance abuse counselor, and another 10 % reduce their intake of alcohol so that they no longer experience adverse consequences from their drinking [23]. A meta-analysis of 23 controlled trials that enrolled persons with alcohol misuse but excluded persons with alcohol dependence indicated that in the primary care setting, the best evidence is for brief (10- to 15-min) repeated behavioral interventions [24]. In adults receiving behavioral interventions, consumption decreased by four drinks per week from baseline, and one-third of patients significantly decreased consumption of alcohol to levels considered safe [24].

In addition to psychological therapies, some patients may benefit from pharmacologic therapy. Currently, four pharmacologic agents have been tested in clinical studies: acamprosate, naltrexone, disulfiram, and baclofen. Also, an extended-release injectable form of naltrexone has been approved to treat alcohol dependence. Data supporting the role of pharmacologic therapies of alcoholism in patients with history of acute AH or advanced cirrhosis are limited due to concern for hepatic toxicity [10]. However, these agents have a modest effect. The only pharmacotherapy evaluated in a randomized controlled trial in patients with advanced ALD is baclofen, a γ-amino butyric acid receptor antagonist. This drug was well tolerated and effective at promoting abstinence in alcohol-dependent patients, resulting in a significant reduction of recidivism compared to placebo (71 % vs. 29 %) with an excellent safety profile [12, 25].

Both acamprosate and naltrexone have been shown to reduce drinking days and increase abstinence rates in randomized controlled trials and in a meta-analysis [26–28]. Acamprosate is well tolerated in most patients except for those

with Child-Pugh C cirrhosis [29], and its benefit seems to persist for at least 1 year after treatment withdrawal. Disulfiram, an inhibitor of acetaldehyde dehydrogenase, induces acetaldehyde-mediated adverse reaction to alcohol intake characterized by nausea and flushing. However, trials of effectiveness have given conflicting results [30, 31].

Nutritional Support

Virtually every patient with AH shows some sign of malnutrition underscoring the importance of nutritional supplementation in the therapy of AH [32–34], with prevalence between 20 and 60 % in outpatients with alcoholic cirrhosis and almost 100 % in hospitalized patients with acute AH [33, 35–38]. The prevalence and severity of malnutrition increase with the amount of alcohol use, lower socioeconomic status, and lack of employment [39, 40]. The presence of malnutrition in patients with AH is associated with significantly higher rates of liver-related complications and mortality, longer ICU stays, and longer durations of hospital stays [34]. In a study of male veterans with severe AH, mortality was more than 80 % in patients with daily intake of less than 1000 kcal/day, whereas almost no mortality was observed in patients with an intake of greater than 3000 kcal/day [32].

The reasons for malnutrition in patients with AH included decreased oral intake and nutrient absorption; derangements of protein, fatty acid, and carbohydrate metabolism; hypermetabolic state; and micronutrient and vitamin deficiencies [34]. The net negative energy balance in AH is further attributable to the microsomal metabolism of ethanol, which is an energy-consuming process [41] and increased gut permeability with low-grade endotoxemia triggering the release of inflammatory cytokines and activation of sympathetic nervous system [42, 43]. Interestingly, a calorimetric study in humans showed that presence of large ascites on admission increases the negative energy balance further by about 10 % [43]. Most patients with AH have underlying cirrhosis, and the lack of glycogen in hepatocytes of cirrhotic livers predisposes these patients to early starvation only after 12 h of fasting compared to 48 h in normal persons [44, 45]. Even short periods of inadequate nutrition trigger muscle proteolysis, contributing to protein malnutrition. Alcohol increases urinary and fecal nitrogen losses, resulting in a net negative nitrogen balance [46]. Not surprisingly, the protein intake recommended for patients with cirrhosis is higher than that for healthy adults [47, 48], and the positive impact of nutritional supplements in patients with cirrhosis is illustrated by a randomized trial that showed that a snack of 700 kcal every evening proved to have a glycogen- and protein-sparing effect and resulted in an accrual of 2 kg of lean tissue over 12 months [44].

Assessment of the nutritional status of patients with AH is difficult. Proteins such as albumin or prealbumin produced in hepatocytes cannot be used for the assessment of nutritional status because their levels correlate with the severity of liver disease rather than malnutrition [49]. Two clinical tools have proved to be useful in patients with AH: the subjective global assessment (SGA) of protein energy malnutrition and measurement of hand grip strength. Both methods have been endorsed by the European Society for Clinical Nutrition and Metabolism as tools for bedside nutritional assessment [48, 50]. SGA is composed of an evaluation of weight trend, dietary intake, physical appearance, and existing medical conditions. Of the various components, muscle wasting is weighted the most and body weight change is the least reliable because of volume shifts frequently seen in these patients [51, 52]. Bedside anthropometry consists of mid arm circumference measurement [53–55] or by evaluating handgrip using Jamar handgrip dynamometer [55, 56]. Muscle strength correlates with malnutrition severity but not with the severity of liver disease, alcohol intake, or neuropathy [57].

Adequate nutritional support is the most frequently overlooked aspect of the management of patients with AH and is critical to the management of patients with severe AH. The goals of nutritional management are to meet basal needs and provide additional sources for hypermetabolic state, and calories should be distributed as

50–60 % to carbohydrates, 25–30 % to proteins, and 15–20 % to fats [34]. The current recommendation is that patient should consume 1–1.5 mg protein per kilogram body weight daily [48]. Previously, it was believed that protein should be restricted in patients with cirrhosis; however, later studies showed that patients restricted of proteins in their diet suffer pronounced muscle catabolism, leading to rise in ammonia and worsening of encephalopathy. Indeed, studies cast doubt on the use of protein restriction even during encephalopathy [58–61].

Early randomized clinical trials comparing nutritional supplements with standard nutrition in patients with AH have shown improvement in nutritional status with enteral supplementation without worsening hepatic encephalopathy, but there was no survival benefit in any of these studies, with mortality between 15 and 40 % regardless of dietary intervention [62–64]. However, neither of these studies used Maddrey's discriminant function >32 as a predefined criterion for severe AH. A review of data presented in these studies indicated that substantial proportion of patients may have had mild or moderate but not severe AH, therefore decreasing the power for detecting significant difference [62–64].

Probably the most compelling data in favor of nutrition therapy came from a multicenter study by Cabre et al. [65] from the Veterans Administration (VA). In this study, patients with severe (DF>32) AH were randomized to receive prednisone, 40 mg daily, or a nutritional formula containing 2000 kcal/day through a feeding tube. The one-month mortality was the same in both groups, but the one-year mortality was significantly lower in the enteral nutrition group compared to the glucocorticoid group, mainly due to fewer infectious complications. Similar results were seen in another cohort of VA male veterans, in which adequate caloric intake (more than 2000 kcal/day) was associated with 19 % mortality, whereas patients with inadequate intake exhibited 51 % mortality at 6 months [63]. Based on these randomized clinical studies, some centers do not hesitate to place a nasogastric feeding tube if the patient cannot ingest at least 2000 kcal/day [33, 48]. Patients with esophageal varices can safely undergo placement of enteral tube without increased risk of variceal hemorrhage [33, 48].

In addition to protein–calorie malnutrition, patients with AH have a plethora of vitamin and mineral deficiencies. Among those, zinc deficiency in patients with AH is of special concern given the requirement of zinc for stabilization of gut barrier function and for attenuation of endotoxemia, pro-inflammatory cytokine production, and hepatocyte apoptosis, all of which represent key features of AH [66]. Zinc supplementation in patients with alcoholic cirrhosis produced beneficial effects on liver metabolic function and liver parameters [67, 68], and clinical study including zinc supplementation in patients with severe AH is ongoing (NCT01809132 at www.clinicaltrials.gov). Addition of *N*-acetylcysteine to enteral nutrition in patients with severe AH did not provide survival benefit [69]. The recommended supplementation regimen for patients with AH is detailed in Table 15.1.

Corticosteroids

Corticosteroids (prednisone or methylprednisolone) represent the most intensely studied pharmacological treatment in AH. They are aimed at suppressing the hepatic inflammatory response through inhibition of nuclear factor κB transcriptional activity [70], which is thought to be a crucial signaling pathway activating innate immune response in alcoholic liver disease [71, 72]. In spite of limitations of available therapeutic trials, corticosteroids are probably the most effective mortality-reducing agents in certain subgroups of patients with AH. Their use was incorporated into the AASLD [3] and EASL (European Association for the Study of Liver) [12] guidelines that recommend to initiate treatment with steroids in patients with severe forms of AH (defined as Maddrey's discriminant function (DF) >32) on the basis of two meta-analyses showing reduction in short-term mortality [73, 74]. There are strengths and limitations of the use of corticosteroids in AH indicated by the diverse selection criteria and study designs used in the evaluation of the therapeutic benefits of

Table 15.1 Nutritional supplementation recommendations for patients with alcoholic liver disease

Energy	25–40 kcal/kg/day, with extra 10 % in patients with ascites
Carbohydrate	• 50–60 % of total calories • Blood glucose monitoring recommended especially in patients treated with corticosteroids
Protein	• 1.0–1.5 g/kg/day even in patients with mild encephalopathy • In patients with grade 3 or 4 hepatic encephalopathy, 0.6–0.8 g/kg/day starting dose recommended with gradual increase • Whole protein formula recommended except patients in grade 3 or 4 encephalopathy when use of branched chain amino acids is recommended
Fat	20–30 % of total energy intake
Fluids	40–50 mL/kg/day, with restriction to 1.0–1.5 L/day if serum sodium <125 mmol/L
Thiamine	100 mg/day, given for at least 2 weeks after hospitalization
Folic acid	1 mg/day, given for at least 2 weeks after hospitalization
Vitamin D	50,000 units three times per week
Vitamin A	10,000 units daily or 50,000 units three times per week
Vitamin E	400 units/day
Iron	Use only if iron deficiency
Calcium	1200–1500 mg/day
Zinc	220 mg twice daily

Adapted from Singal and Charlton [34], with permission of Elsevier

corticosteroids. The phrase from Dr. Charles Davidson, "prednisolone survival effect becomes evident only in a group of patients [with AH] neither so ill that their fate is already sealed nor yet so well that they would recover anyway," well portrays the limited patient population where corticosteroids might be beneficial [75–77].

Review of Studies of Corticosteroids in Patients with AH

The therapeutic use of corticosteroids in AH has to be viewed from the perspective of trials in which their effect was evaluated. These trials have numerous limitations, including the heterogeneity of patients with AH, presence of nonresponders to corticosteroids, and exclusion of patients with infections, GI bleeding, or renal insufficiency.

The "Steroid Controversy"

AH is associated with significant inpatient mortality that can reach up to 80 % in the most severe forms [78]. The main causes of death are liver failure, sepsis, hepatorenal syndrome, and gastrointestinal bleeding. Evaluation of treatment effect on short-term survival requires identification of patients with significant risk of death. Until the Discriminant Function became available [78, 79], no objective and reproducible criterion existed to predict early mortality. The heterogeneity of patients with AH that were enrolled in corticosteroid trials in pre-DF era resulted in a wide range of survival in the placebo-treated arms, ranging from 81 % if patients with mild AH were included to 0 % survival in trials with patients with the most severe AH [76–83].

The DF described by Maddrey et al. identifies patients with a high risk of early (1–2-month) mortality [79, 81]. It is calculated as 4.6 (prothrombin time − control prothrombin time [in seconds] + bilirubin [in μmol/L]/17). The presence of DF > 32 and/or encephalopathy is used as a definition criteria for acute AH, which, in the absence of treatment, has spontaneous survival between 50 and 65 % [78, 79, 81]. After acceptance of DF > 32 as inclusion criteria of patients with AH to the clinical trials, the short-term survival of glucocorticoid-treated patients has remained remarkably constant, with a 2-month survival rate of approximately 80 % [73, 79, 84–86].

The variable severity of AH in patients enrolled into clinical trials was likely the strongest contributor to the "controversial evidence" of the efficacy of steroids in AH, advocated by some authors [87–89]. This skepticism was further deepened by the widespread belief in the 1970s and 1980s that corticosteroids caused duodenal ulcers [90] and by the belief that corticosteroids increase the risk of infection [91]. The latter

myths have been recently disproved by two randomized controlled trials ([91, 92] and below), and the "steroid controversy" has been the subject of multiple meta-analyses [73, 74, 89, 93, 94] and of an ongoing prospective trial [88].

The attempts to clarify whether corticosteroids improve survival in AH followed two approaches. The first approach relied on the classical methodology of meta-analysis utilizing published results of randomized controlled trials [89, 93, 94]. The second approach was based on a meta-analysis of primary, raw data derived from randomized controlled trials but restricted only to patients with severe alcoholic hepatitis (i.e., those having DF > 32 and/or encephalopathy) [73, 74].

In 1990, Imperiale et al. [93] studied 11 randomized trials from 1966 to 1989 and found that patients with alcoholic hepatitis treated with steroids were associated with a 36 % (CI 95 %, 28–50 %) reduction in mortality. In this meta-analysis, the mortality benefit was only evident in patients with hepatic encephalopathy, and the influence of admission DF on therapeutic effect of steroids was not evaluated in this study [93]. In 1994, Christensen et al. [94] examined 13 randomized controlled trials published between 1971 and 1992 and found no survival benefit of therapy of corticosteroids. In this meta-analysis, Christensen et al. did not perform a subgroup analysis of the data [94], but a review of the studies included in that meta-analysis indicates the survival benefit of steroids in all three studies in which Maddrey's DF was used as inclusion criteria (studies [78, 79, 81] within meta-analysis [94]). This study was followed by another meta-analysis from the same group, published in 2008 under the label of the Cochrane Hepato-Biliary consortium [89] that examined 15 randomized trials with a total of 721 patients. The study found that corticosteroids did not reduce mortality of the entire population of patients with alcoholic hepatitis, compared with placebo, but mortality was statistically reduced in subgroups that were either experiencing hepatic encephalopathy or could be characterized as having severe AH with DF > 32 [89]. In spite of the beneficial effect of corticosteroids in patients with severe AH (as opposed to patients with mild or moderate AH), both meta-analyses [89, 94] lead Christensen et al. to conclude that corticosteroids are ineffective in patients with AH [94].The appropriateness of this conclusion was challenged by Imperiale, O'Connor, and McCullough [95] who emphasized the beneficial effect of corticosteroids in patients with severe alcoholic hepatitis.

In 2001, Mathurin et al. [73] used pooled primary data from three placebo-controlled trials to compare corticosteroids with placebo in patients, all of whom had severe AH (DF > 32). In this group of patients, at 28 days, patients who received corticosteroids had a significantly higher survival (85 % vs. 65 %) compared to those who received placebo, revealing that steroid treatment of severe alcoholic hepatitis is associated with a survival advantage, with a number needed to treat of 5. Increasing age and serum creatinine were found to be independent prognostic factors for death from severe AH in this study [73]. Nine years later, Mathurin et al. published another meta-analysis [74] based on individual patient data by adding two additional randomized controlled trials [65, 96] to their previous analysis [73]. Again, only data for patients with DF > 32 on admission were included in meta-analysis. Combination of all five RCTs [65, 79, 81, 96, 97] confirmed beneficial effects of corticosteroids on short-term survival, demonstrating an 80 % survival of patients treated with steroids at one month, compared to 65 % survival in patients treated with placebo [74].

Nonresponders to Corticosteroid Treatment

About one-fourth of patients with severe AH (DF > 32) will not respond to corticosteroids [85]. These patients have a grim prognosis, with fewer than 25 % surviving 6 months. The benefits of early identification of nonresponders to steroid treatment would be that these patients could be spared of side effects of unnecessary corticosteroid treatment and that alternative therapies, such as biological treatment or liver transplant, advocated by some authors [98, 99], could be considered.

Nonresponsiveness to steroids can be predicted in an assay that tests responsiveness to

prednisone in peripheral blood mononuclear cells isolated from patients with AH [100]. In this assay, termed DILPA (dexamethasone suppression of lymphocyte proliferation test), lymphocytes isolated from the blood of patients with severe AH are stimulated with phytohemagglutinin in vitro with or without the presence of dexamethasone [100]. The assay duration is about 48 h and requires ^{3}H thymidine for the testing of lymphocyte proliferation. A lack of response to steroids is defined as suppression of lymphocyte proliferation by less than 60 % of the maximal proliferation count. The accuracy of the DILPA assay in predicting 6-month survival in patients with severe AH is 0.86 (as determined by area under the ROC) [101]. The DILPA assay has not been widely accepted in clinical practice.

Out of clinical parameters that would predict responsive to corticosteroids, the early change in bilirubin levels (ECBL, defined as bilirubin level at 7 days lower than bilirubin level on the first day of treatment) has shown high predictive value [85]. In their 2003 prospective study involving 238 patients, Mathurin et al. [85] set out to identify clinical and laboratory markers that would predict lack of response to corticosteroids in patients with severe AH (DF > 32). They used 6-month survival as an end point because of the rule requiring 6 months for listing alcoholic patients for liver transplantation. All patients were treated with corticosteroids. The overall 1-month survival was 85 %, consistent with previous data on survival in corticosteroid-treated patients with AH [73, 74], and the 6-month survival was 64 %. An ECBL at 7 days was observed in 75 % of patients. At 6 months, survival of patients with ECBL (83 %) was significantly higher than in patients without ECBL (23 %). ECBL was by far the strongest predictor of 6-month survival (odds ratio 7.1 [95 % confidence interval 4.0–11.1]) for 6-month survival compared to patients with no ECBL. The study suggested that evolution of bilirubin level at 7 days may be useful in identification of severe AH patients with poor prognosis, and the predictive value of ECBL was higher than that of PT or DF.

The ECBL has been incorporated as a dynamic component into the Lille model predicting the 6-month survival in patients with severe AH [102]. Other clinical predictors included in the Lille model are age, the presence of renal insufficiency, albumin level, and prothrombin time. The Lille score calculator (available at http://www.lillemodel.com/score.asp) generates a score that ranges between 0 and 1. The ideal cutoff of 0.45 can be used to define responders to corticosteroids (Lille score < 0.45) and nonresponders (Lille score > 0.45). The therapeutic response to steroids can be further refined by defining three populations: complete responders (Lille score < 0.16), partial responders (Lille score 0.16–0.56), and null responders (Lille score > 0.56) [98]. This sub-stratification has prognostic value, as demonstrated in two subsequent studies by Mathurin's group [92, 103]: the 6-month survival is about 90 % in complete responders, 45–78 % for partial responders, and less than 45 % in null responders. DF, MELD, or Glasgow score have significantly worse accuracy to predict 6-month survival, compared to the Lille score (are under the ROC of 0.73, 0.72, and 0.67, respectively, vs. 0.85 for Lille score) [102]. In patients with Lille score > 0.45 (nonresponders), the EASL guidelines recommend to discontinue corticosteroids after 7 days of treatment [12].

Steroids in AH Patients with Infection: Timing Matters

Infection has long been considered a contraindication to glucocorticoid therapy in patients with severe alcoholic hepatitis. It is estimated that the presence of infection as exclusion criterion in patients with severe AH may have barred 25 % of otherwise eligible patients from consideration of treatment [91]. It is thought that liver failure with resulting immune paralysis is a major factor driving increased susceptibility to bacterial infections in patients with advanced liver cirrhosis or severe AH [104, 105].

In their prospective study, Louvet et al. [91] performed screening for infection in 246 patients admitted for severe AH. All patients underwent chest X-ray and their blood, urine, and ascites were cultured. Out of all patients, 63 (25 %) were found to have an infection (spontaneous bacterial

peritonitis, urinary tract infection, and pneumonia being the most prevalent infections) and received antibiotic treatment. Corticosteroids were started only after a priori defined criteria for antibiotic treatment response were met. Patients with severe AH and with infection responding to antibiotic treatment had similar 2-month survival compared to patients with severe AH and no infection (71 % vs. 72 %). Data on 6-month survival were not provided in the study [91].

However, there was a significant difference in survival when patients with infection treated prior to initiation of steroids (above) were compared to patients who developed infection while on treatment with steroids. After initiation of corticosteroids, 57 patients (24 %) developed an infection after a median time of 14 days, and there has been substantial proportional increase in pneumonia (40 % of infected patients). Patients infected after corticosteroid treatment had significantly lower 2-month survival (46 %) compared to noninfected patients (78 %). Only the Lille score and MELD score were independent predictors of survival in these patients, whereas ascites, encephalopathy, Maddrey DF, leukocytosis, or C-reactive protein levels were not. Importantly, the presence of infection after corticosteroid initiation proved to be a negative determinant of survival only in patients who responded to corticosteroids (i.e., Lille score <0.45), but not in nonresponders (Lille score >0.45) where it is thought that it was the lack of steroid response but not infection determining unfavorable prognosis in nonresponders [91].

This study suggested that it is safe to use corticosteroids in patients with severe AH if infection is identified and treated *prior* to initiations of corticosteroids. The study also showed that survival benefit of antibiotic therapy in patients who develop infection *after* initiation of corticosteroid treatment can be expected only in responders to steroids [91].

Steroids in AH Patients with GI Bleeding

Both AASLD and EASL guidelines specify that patients with severe AH with recent upper GI bleed are not ideal candidates for corticosteroid treatment [3, 12]. The reason for this may be a carryover from clinical trials performed in the 1970s and 1980s in which patients with upper GI bleeding were excluded due to the belief that corticosteroids caused gastroduodenal ulcers and also because no effective treatment of upper GI bleeding existed at that time [90]. Since then, however, much has changed, including the advent of proton pump inhibitors, endoscopic treatment of variceal bleeding, and transjugular portosystemic shunting [106]. Also, antibiotics given for 7 days after GI bleeding in patients with cirrhosis reduce infections and increase survival [107].

Rudler et al. [92] conducted a retrospective analysis of survival among patients with severe AH who presented to a hospital with upper GI bleed and compared them with patients with severe AH without GI bleeding. A total of 48 patients with upper GI bleed and 47 patients without GI bleed were analyzed. The two groups did not differ in the presence of AH on biopsy (approximately 80 %) and DF or MELD score. After stabilization and effective bleeding control per Baveno V recommendations [108], both groups were started on corticosteroids. The 6-month survival was similar in both groups (74 % vs. 70 %). The probability of developing an infection after starting corticosteroids was lower among subjects with upper GI bleed (24 %) as compared with subjects without upper GI bleed (45 %). This was attributable to antibiotic therapy mandated in patients with acute GI bleed and could have improved survival in GI bleeders in the study. If validated in prospective trials, this data indicate that GI bleed does not worsen survival in AH patients treated with corticosteroids [92].

Pentoxifylline

In a randomized controlled trial in severe AH, treatment with pentoxifylline improved survival compared to placebo [109]. Two other studies have evaluated pentoxifylline in AH, in smaller cohorts and without histological confirmation of AH. In a study comparing pentoxifylline with corticosteroids, a benefit in survival in the pentoxifylline group was observed, with 15 % 1-month mortality in pentoxifylline-treated

patients vs. 35 % mortality in patients treated with corticosteroids [110]. In a second study, in which a combination of pentoxifylline and corticosteroids was compared with corticosteroids alone, no difference in survival was found; however, this study was not double-blind in design [111]. Experimental data have demonstrated the anti-inflammatory and antitumor necrosis factor alpha effects of pentoxifylline [112], although its benefit in severe alcoholic hepatitis seems to be related to the prevention of hepatorenal syndrome [103, 109]. In the subgroup of patients not responding to corticosteroids, defined as the lack of early change in bilirubin level on day 7, corticosteroids were discontinued and pentoxifylline was started; however, this proved to be an ineffective rescue strategy, resulting in 36 % 2-month survival in nonresponders in whom corticosteroids were discontinued, compared to 31 % 2-month survival in nonresponders transitioned from corticosteroids to pentoxifylline [113].

A large randomized controlled trial in 270 patients with biopsy-proven, severe AH did not show any benefit in survival with the combined administration of pentoxifylline and corticosteroids, compared to corticosteroids alone; a lower incidence of hepatorenal syndrome was noted in the combined treatment group (3 % vs. 12 % in corticosteroid group at 1 month, although this difference was no longer significant at 6 months) but did not result in improved survival compared to the prednisolone-alone group [103]. In the large randomized controlled trial STOPAH, which included more than 1000 patients [88], results with pentoxifylline were not better than placebo for short-term mortality [98, 114]. In summary, the data available to date does not provide strong evidence to support using pentoxifylline in ALD, and there is no role of pentoxifylline as adjunct or rescue therapy in patients treated with corticosteroids.

Liver Transplantation

According to UNOS, history of alcohol use as the etiology for cirrhosis in patients with end-stage liver disease represents 18.7 % of those who receive liver transplantation in the United States (http://optn.transplant.hrsa.gov/converge/data/default.asp) [115]. All of these patients on liver transplant lists are expected to demonstrate at least 6 months of alcohol-free period and a strong support system to prevent relapse in alcohol use. Survival of the transplanted patients and organs is excellent in this patient population [116]. Five-year patient survival of alcoholic hepatitis and alcoholic cirrhosis was 80 % and 78 %, respectively, and five-year graft survival was 75 % and 73 % [116]. In contrast to those who stopped alcohol, patients with acute alcoholic hepatitis who fail to respond to medical therapy are not considered for liver transplantation in most transplant centers. A recent study from Europe demonstrated that early liver transplantation resulted in improved cumulative 6-month survival and that benefit was maintained during the 2-years follow-up. Of the transplanted 26 patients, 3 had alcohol use relapse after transplantation [99]. Given the shortage of livers for transplantation and the addictive behavior of patients with alcoholic hepatitis, there are many ethical considerations regarding liver transplantation in patients with severe acute alcoholic hepatitis [117]. Investigation of the utility of liver transplantation for patients with severe steroid-resistant alcoholic hepatitis is an area where future clinical trials are needed.

Emerging and Novel Therapies in Alcoholic Liver Disease and Alcoholic Hepatitis

Standard of care in alcoholic liver disease and alcoholic hepatitis is very limited and are based on old studies and recommendations from decades ago. The lack of efficient treatment options clearly demonstrate that alcoholic liver disease has been a “neglected” disease with respect to clinical trials, drug development, and attention of the pharmaceutical industry and clinical research. This devastating picture has been somewhat modified by the recent initiative of the NIAAA that initiated clinical and translational research in alcoholic hepatitis.

When approaching treatment of alcoholic liver disease and/or alcoholic hepatitis, understanding critical elements of disease pathomechanisms is critical and could guide the design of disease-specific interventions. Studies in animal models indicate many potential checkpoints for targeting the development of alcoholic liver disease.

These include but are not limited to gut permeability, hepatocyte damage and cell death, mediators and cell types of inflammation, process of fibrosis, regeneration, and hepatocellular cancer. Emerging therapies will be discussed based on these categories below. There are several important considerations in designing new therapeutic interventions:

- What is the status of the disease induced by alcohol? Treatment of moderate and acute severe alcoholic hepatitis will very likely require different approaches and interventions. Although the exact triggers and mechanisms that differentiate moderate from severe alcoholic hepatitis are yet to be delineated, experimental data suggest that the severity of alcoholic liver disease is determined not only by quantitative but also qualitative differences in the pathomechanism. For example, in severe acute alcoholic hepatitis, activation of innate immune responses and the pro-inflammatory cytokine cascade in severe alcoholic hepatitis represents a vicious cycle that might be very difficult to break without compromising fundamental elements of host defense.
- Will one agent/drug be sufficient to interfere with the multifactorial process in the pathogenesis of acute alcoholic hepatitis? Consideration of therapeutic approaches that target different key components of acute alcoholic hepatitis may be more effective than a single therapy.

Targeting the Gut

Based on recent experimental evidence on the effect of alcohol on the gut, interventions that prevent alcohol-induced increase in gut permeability and microbial translocation and/or restore alcohol-induced disturbance of the gut microbiome could be reasonable interventions. It remains to be evaluated whether these components should be targeted individually or collectively to achieve improvement in alcoholic liver disease or in alcoholic hepatitis [118, 119]. Animal and human studies identified the benefit of zinc on gut permeability [120]. Targeting the microbiome composition by using VSL3 or lactobacillus GG may have benefits in alcoholic liver disease [121, 123]. Fecal transplantation has proven benefit in *Clostridium difficile* infection, and it is in early clinical trials in alcoholic liver disease [122]. Additional potential targets based on animal studies include miR-155 inhibition [124]. Farnsenoid receptor (FXR) activation inhibited inflammation and preserved the intestinal barrier in inflammatory bowel disease, and its potential role in alcoholic liver disease is yet to be explored [125] (Fig. 15.1).

Modulation of Steatosis

Alcohol-induced steatosis in hepatocytes is an early effect of alcohol that is sustained during chronic alcohol use. Of the many signaling mechanisms regulating hepatocytes fat accumulation, activation of the PPAR-alpha was shown to have benefits on murine alcohol-induced liver disease [126]. PPAR-gamma and PPAR-delta agonist treatment improved hepatic insulin resistance in another study [126].

Modulation of Inflammation and Immunity

Recruitment of macrophages and neutrophils to the liver in alcoholic liver disease is mediated by chemokines [127]. MCP-1 (also named CXCL2) is produced by alcohol-exposed hepatocytes and immune cells are increased in early alcoholic liver disease and they recruit monocytic cells into the liver. In addition, MCP-1 induces fat accumulation in hepatocytes [128, 129]. In mice deficient of MCP-1, chronic alcohol feeding resulted in

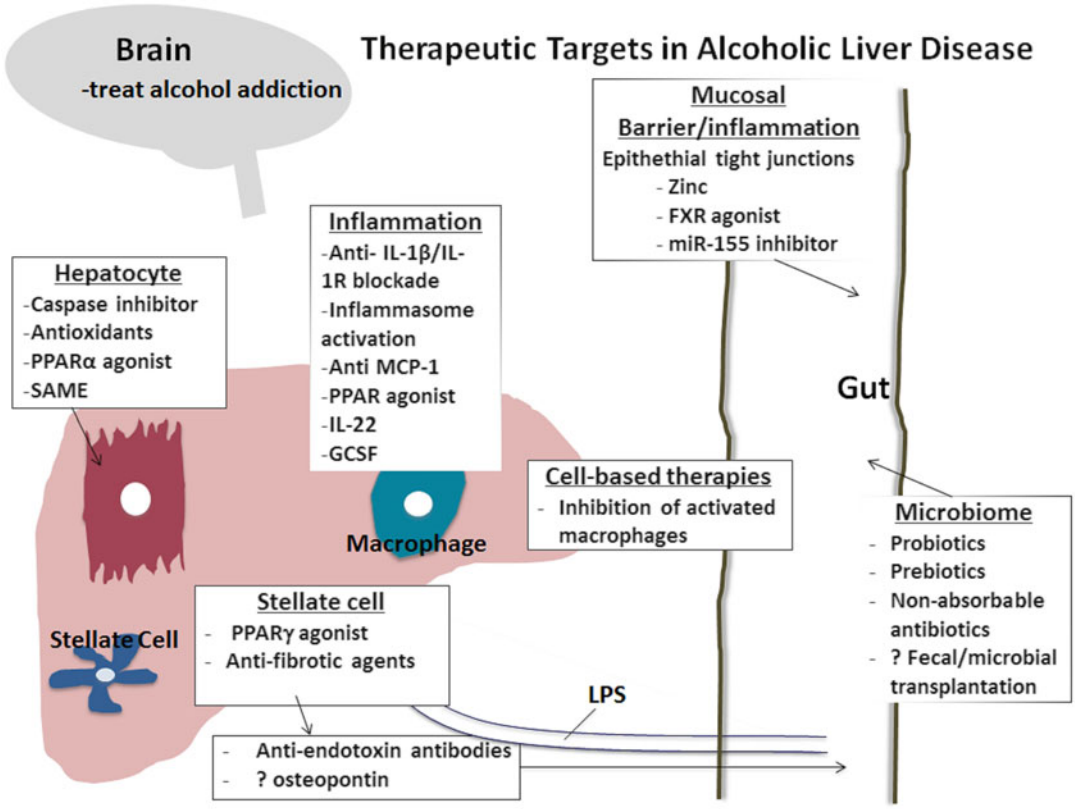

Fig. 15.1 Emerging and novel targets in treatment of alcoholic liver disease and/or alcoholic hepatitis

attenuated steatosis and inflammation suggesting that inhibition of MCP-1 may provide benefits in ALD. A dual CCR2/CCR5 antagonist cenicriviroc, is currently in clinical trial in human nonalcoholic steatohepatitis and testing of this antibody in ALD awaits investigation.

Targeting pro-inflammatory cytokines in acute alcoholic hepatitis has been the target of previous and current investigations. Clinical trials using anti-TNF-α blocking antibodies or inhibiting TNF receptor I had unsuccessful outcomes due to increased infections [130–133]. While TNF has complex effects on the immune system and the liver, studies with anti-TNF-α are perfect reminders about the impaired immune system of patients with alcoholic liver disease. Alcohol compromises both innate and adaptive immunity and the increased pro-inflammatory cascade activation in acute alcoholic hepatitis occurs together with impaired immune responses in these patients [134]. It remains to be seen whether anti-inflammatory strategies alone or in combination with other treatment could be beneficial. Currently, clinical trials have been initiated with the combination of recombinant IL-1 receptor antagonist combined with zinc and pentoxifylline in severe acute alcoholic hepatitis. Another study will test the effects of an anti-IL-1 antibody in combination with steroids in severe alcoholic hepatitis (Table 15.2).

Based on animal data and observations in human monocytes, the delivery of cell-specific therapies deserves further exploration. For example, ample evidence suggests that activated Kupffer cells and macrophages could be attractive therapeutic targets in the liver to reduce inflammation. However, macrophage-specific delivery methods are yet to be optimized. Potential targets in macrophages include NF-kB, microRNA-155, and ERK1 signaling (Fig. 15.1) [5, 135].

While treatment with biologics has been successful in rheumatologic and inflammatory

Table 15.2 Alcoholic hepatitis clinical trials data

Title	Recruitment phase	Conditions
Effect of Probiotics on Gut-Liver Axis of Alcoholic Hepatitis	Active, not recruiting	Alcoholic liver disease
Pharmacokinetic and Pharmacodynamic Study of IDN-6556 in ACLF	Active, not recruiting	Acute-on-chronic hepatic failure | acute liver failure | liver cirrhosis | acute alcoholic hepatitis
Assess Safety and Efficacy of ELAD (Extracorporeal Liver Assist System) in Subjects With Alcohol-Induced Liver Failure	Active, not recruiting	Acute alcoholic hepatitis
Short-term Survival in Patients With Severe Alcoholic Hepatitis Treated With Steroid Versus Pentoxifylline	Enrolling by invitation	Alcoholic hepatitis
FGL2/Fibroleukin and Hepatitis C Virus Recurrence Post Liver Transplantation	Enrolling by invitation	Liver transplantation | hepatitis C
Efficacy of Antibiotic Therapy in Severe Alcoholic Hepatitis Treated With Prednisolone	Not yet recruiting	Alcoholic hepatitis | alcoholic liver disease
Randomised Open-label Multicenter Study Evaluating Ciprofloxacin in Severe Alcoholic Hepatitis	Not yet recruiting	Alcoholic hepatitis | alcoholic cirrhosis
A Safety and Efficacy Study of Mycophenolate Mofetil and Rilonacept in Patients With Alcoholic Hepatitis	Not yet recruiting	Alcoholic hepatitis
Safety and Efficacy of IMM 124-E for Patients With Severe Alcoholic Hepatitis	Not yet recruiting	Alcoholic hepatitis
Validation of the Procedure of Early Liver Transplantation in Alcoholic Hepatitis Resisting to Medical Treatment	Recruiting	Alcoholic hepatitis | alcoholic cirrhosis
Protective Immune Mechanisms in Alcoholic Hepatitis	Recruiting	Alcoholic hepatitis
Effects of Prednisolone and Pentoxifylline on the Regulation of Urea Synthesis in Alcoholic Hepatitis	Recruiting	Alcoholic hepatitis
Study to Assess Safety and Efficacy of ELAD in Subjects With Severe Acute Alcoholic Hepatitis (sAAH) and Lille Score Failure	Recruiting	Severe acute alcoholic hepatitis
Novel Therapies in Moderately Severe Acute Alcoholic Hepatitis	Recruiting	Acute alcoholic hepatitis
Effects of Rifaximin in Patients With Acute Alcoholic Hepatitis	Recruiting	Alcoholic hepatitis
Integrated Approaches for Identifying Molecular Targets in Alcoholic Hepatitis	Recruiting	Alcoholic hepatitis
Efficacy of G-CSF in the Management of Steroid Non-responsive Severe Alcoholic Hepatitis	Recruiting	Severe alcoholic hepatitis
Immune Cell Dysfunction in Severe Alcoholic Hepatitis	Recruiting	Hepatitis
Alcohol Diet and Drug Use Preceding Alcoholic Hepatitis	Recruiting	Alcoholic hepatitis
Alcoholic Hepatitis: A Multicenter, Observational Study by the TREAT Consortium	Recruiting	Alcoholic hepatitis
Trial of Obeticholic Acid in Patients With Moderately Severe Alcoholic Hepatitis (AH)	Recruiting	Alcoholic hepatitis
Efficacy and Safety of MG in the Patients With Alcoholic Fatty Liver Disease and Alcoholic Hepatitis	Recruiting	Alcoholic fatty liver disease | alcoholic hepatitis

(continued)

Table 15.2 (continued)

Title	Recruitment phase	Conditions
Granulocyte Colony Stimulating Factor (G-CSF) in Acute Liver Failure and Alcoholic Hepatitis	Recruiting	Acute liver failure
Efficacy Study of Anakinra, Pentoxifylline, and Zinc Compared to Methylprednisolone in Severe Acute Alcoholic Hepatitis	Recruiting	Acute alcoholic hepatitis
Efficacy and Safety of S-adenosyl-l-methionine in Treatment of Alcoholic Hepatitis With Cholestasis	Recruiting	Alcoholic hepatitis
The Effect of High Dose Vitamin C on the Liver Function in Chronic Hepatitis Patients	Recruiting	Chronic hepatitis | chronic hepatitis C | chronic alcoholic hepatitis
National Cohort of Uncomplicated Alcoholic Cirrhosis	Recruiting	Alcoholic cirrhosis
Acoustic Liver Biopsy in Normals and in Patients With Cirrhosis Using Endoscopic Ultrasound	Recruiting	Normal | alcoholism | cirrhosis | hepatitis C
Integrated Stepped Care for Unhealthy Alcohol Use in HIV	Recruiting	Liver diseases, alcoholic | alcoholism | HIV | hepatitis C
Transient Elastography in the Determination of Advanced Fibrosis in Alcoholic Liver Disease	Recruiting	Alcoholism | liver disease | liver fibrosis
N-Acetylcysteine in Severe Acute Alcoholic Hepatitis	Completed	Alcoholic hepatitis
Inflammation, Immune Activation and Portal Hypertension in Alcoholic Hepatitis	Completed	Alcoholic hepatitis
Metadoxine as a Therapy for Severe Alcoholic Hepatitis	Completed	Severe alcoholic hepatitis
Treatment of Severe Alcoholic Hepatitis With Corticoids Plus N Acetyl Cysteine Versus Corticoids Alone	Completed	Alcoholic hepatitis
Efficacy of Combination Therapy of Glucocorticoids and Bovine Colostrum in Treatment of Severe Alcoholic Hepatitis	Completed	Severe alcoholic hepatitis in "extremis"—defined by mDF>54
Intensive Enteral Nutrition and Acute Alcoholic Hepatitis	Completed	Severe alcoholic hepatitis
Double-blind Randomized Controlled Trial in Severe Alcoholic Hepatitis	Completed	Alcoholic hepatitis | alcoholic liver disease
Adipose Tissue Involvement in Alcohol-induced Liver Inflammation in Human	Completed	Alcoholic hepatitis | alcoholic cirrhosis
Randomized, Controlled Trial of S-adenosylmethionine in Alcoholic Liver Disease	Completed	Alcoholic hepatitis
Effect of Probiotics on Gut-Liver Axis of Alcoholic Liver Disease	Completed	Alcoholic liver disease
Efficacy and Safety of the Extracorporeal Liver Assist Device (ELAD) in Acute on Chronic Hepatitis	Completed	Acute-on-chronic hepatitis
Role of CCL2 in Alcoholic Liver Diseases	Completed	Alcoholic liver disease
Study of T Cell Phenotype Activation Pathway in Human Alcoholic Liver Disease	Completed	Alcoholic liver disease | chronic hepatitis C virus
FIBROSCAN Validation and Interest of Fibrotest—FIBROSCAN Association for Fibrosis Diagnosis in Alcoholic Liver Disease	Completed	Alcoholic liver disease
Liver Transplantation in Alcoholic Hepatitis	Suspended	Alcoholic hepatitis
Pentoxifylline for Acute Alcoholic Hepatitis (AAH)	Terminated	Alcoholic hepatitis
Study of IDN-6556 in Patients With Severe AH and Contradictions to Steroid Therapy	Terminated	Alcoholic hepatitis
Treatment of Alcohol-Related Hepatitis With Arginine	Withdrawn	Alcoholic hepatitis

Accessed from www.clinicaltrials.gov on April 3, 2015

bowel diseases that have cytokine aberrations similar to ALD, biologics are yet to be explored in alcoholic liver disease. Interleukin-22 is a cytokine that regulates immune functions and also acts on the integrity of the gut mucosa [136]. In a murine model of alcoholic liver disease, IL-22 treatment ameliorated alcoholic liver injury via the activation of hepatic STAT3 and ameliorated alcoholic fatty liver, liver injury, and hepatic oxidative stress [137]. Based on beneficial effects in a mouse model, there are now plans to use IL-22 in an early clinical trial in humans with alcoholic liver disease.

Liver inflammation in acute alcoholic steatohepatitis is characterized by the recruitment of neutrophil leukocytes to the liver, and it has been suggested that increased neutrophil count on liver biopsies correlates with survival. Please note that Bataller's study [19] showed that the degree of neutrophilic infiltration positively correlates with survival. Neutrophil dysfunction is also well documented in alcoholic liver disease [105]. The role of neutrophils in the liver in alcoholic hepatitis is unclear, however, because neutrophils can have dual functions including both the production of harmful ROS as well as participation in tissue remodeling. Interestingly, the administration of G-GSF with standard medical therapy to patients with severe alcoholic hepatitis in a small clinical trial resulted in a significant reduction in median change in CTP, MELD, and mDF at 1, 2, and 3 months. There was also a significant improvement in survival in the standard therapy plus G-CSF group compared to standard therapy alone [138]. These clinical improvements correlated with an increase in the CD34+ cell population in the peripheral blood, indicating recruitment of bone marrow derived cells [138].

Interfering with Hepatocyte Cell Death Pathways

Multiple metabolic signals can induce hepatocytes apoptosis and death in alcoholic liver disease. Studies demonstrated the importance of ROS resulting from alcohol metabolism in hepatocytes and various antioxidants have been tested in previous studies including *N*-acetylcysteine and *S*-adenosyl-L-methionine (SAME) [139, 140]. The administration of NAC plus glucocorticoids in severe alcoholic hepatitis resulted in no significant changes in survival at 6 months but improved mortality at 1 month [141]. Multivariate analysis revealed that younger age, lower bilirubin at baseline, and a decrease in bilirubin on day 14 correlated with better outcomes in the NAC+prednisolone group, compared to prednisolone alone.

Induction of caspases in alcoholic hepatitis has been demonstrated in multiple studies and our group found that mitochondrial hepatocyte death is mediated by IRF3 phosphorylation by the endoplasmic reticulum adapter molecule, STING [142]. ER dysfunction and key elements of autophagy are also targets of chronic alcohol [143]. Thus, it is tempting to speculate that selective caspase inhibitors or agents that improve ER functions would be beneficial in alcoholic liver disease.

Other Novel Therapies in Alcoholic Hepatitis

Overexpression of osteopontin was found in both human and mouse livers with alcoholic hepatitis [144, 145]. While the complex effects of osteopontin are yet to be dissected in alcoholic hepatitis, it appears to be a mediator of hepatic stellate cell activation in alcoholic liver disease [144]. Extracorporeal liver support is increasingly used in acute liver failures including acute alcoholic hepatitis. This has been recently reviewed [146], and further clinical trial should address whether extracorporeal filtration could be useful in recovery of the organ in acute severe AH or just provide a bridge to transplantation or other treatment.

Barriers in Clinical Trial Design and Clinical Trial Implementation in the Patient Population with Alcoholic Liver Disease

Compared to many other liver diseases, there are multiple barriers in development of effective therapy and new interventions in patients with

alcoholic liver disease. First, there is an immediate need for uniformly used and sufficient definition of the different stages of alcoholic liver disease. This would achieve a desirable "homogenous" patient population for clinical trials. A better definition of alcoholic steatosis alone, moderate alcoholic steatohepatitis, acute severe alcoholic hepatitis, and acute-on-chronic alcoholic liver disease is needed based on parameters of clinical presentation and/or liver pathology. Second, endpoint definitions should be developed for alcoholic liver disease that could be uniformly used in clinical trials and be accepted by regulatory agencies. Third, a major limitation in industry-supported clinical trials for alcoholic liver disease is the social "stigma" of alcohol misuse and addiction. Fourth, alcohol addiction that is present in some but not all patients with alcoholic liver disease creates an extra challenge in adherence to therapy and follow-ups in clinical trials designed for these patients. Finally, the severity of patients in the acute alcoholic hepatitis category is another challenge given the high mortality of these patients.

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Current and Emerging Therapies for Nonalcoholic Fatty Liver Disease

16

Samer Gawrieh and Naga Chalasani

Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
ALT	Alanine aminotransferase
HOMA	Homeostasis Model Assessment
TZDs	Thiazolidinediones
OCA	Obeticholic acid
CVD	Cardiovascular disease
ESLD	End-stage liver disease
HCC	Hepatocellular carcinoma

Goals of Treatment

The three most common causes of mortality in patients with NAFLD are cardiovascular disease (CVD), extrahepatic malignancy, and end-stage liver disease (ESLD). To improve patient survival, efforts should be directed at preventing these major events. In this chapter, we will focus on current and emergent therapies to prevent NAFLD progression to ESLD in addition to discussing management options for NAFLD patients with ESLD and hepatocellular carcinoma (HCC). Liver-related aspects of preventing CVD will be discussed. Interventions to reduce the risk of extrahepatic malignancy fall beyond the scope of this chapter, and will not be discussed.

Because the majority of liver-related events are the result of progression of NASH to cirrhosis, liver failure, and HCC, liver-directed therapies have been directed at stopping the progression of or reversing NASH. In patients with liver failure or HCC, liver transplantation should be considered as an option to improve patient's survival and quality of life. While a majority of patients with nonalcoholic fatty liver (NAFL) have a benign hepatic outcome, there are emerging data to suggest that metabolic disturbances associated with NAFLD may improve or reverse by resolution of hepatic steatosis. Given that CVD is the most common cause of death in patients with NAFLD, it is important to optimize the management of established risk factors for CVD including the use of statins to achieve target lipoprotein levels. Emerging therapies including molecules against new targets and anti-fibrotic agents will be discussed.

Liver-Directed Therapies

In the following sections, therapeutic strategies directed at improving liver involvement with NAFLD will be discussed.

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Weight Loss

In numerous clinical studies, mild to moderate weight loss has been shown to improve insulin sensitivity, liver transaminases, hepatic steatosis by various imaging modalities, and liver histology in patient who had NASH [1–19].

The effects of weight reduction on hepatic steatosis can be seen in as early as 2 weeks following weight loss [10]. Subjects losing about 4 % of their baseline weight in 2 weeks with caloric- or carbohydrate-restricted diets demonstrate nearly 40 % reduction in intrahepatic triglycerides [10].

The amount of weight loss necessary to improve imaging-detected hepatic steatosis can be as low as 3–5 % of starting body weight [10, 15]. However, more weight loss may be necessary to improve other lesions associated with NASH. Several small studies examined the effects of non-bariatric weight loss on liver histology in patients with NAFLD [3, 8, 17, 18, 20, 21]. In a study of 25 subjects [17], 15 subjects were assigned to reduced caloric diet plus daily exercise (walking and running) for 3 months. The ten subjects in the control group did not change their diet or physical activity. All participants underwent liver biopsy at the beginning and conclusion of the study. At the end of the study, BMI, ALT, fasting glucose, and steatosis significantly improved in the intervention group. Another study randomized 31 patients with biopsy-proven NASH to receive 48 weeks of dietary modification (weight-based caloric restriction with 25 % fat) and exercise (to accumulate at least 10,000 ft with a pedometer with additional physical activities encouraged), in addition to behavioral strategies to induce and maintain the diet and exercise pattern ($n=21$) [8]. The control group ($n=10$) received only standard education about the diagnosis of NASH, healthy diet, and exercise. In the treatment group, subjects lost a mean of 9.3 % of their baseline weight compared to 0.2 % in controls. In addition to improvement in ALT, there was a significant improvement in the NAFLD activity score in the treatment group. Subjects who lost ≥7 % of baseline weight experienced significant improvement in steatosis, lobular inflammation, and ballooning. There was no change in the mean fibrosis score before and after lifestyle changes in this study. Other studies combined orlistat [20, 21], vitamins E and C [3], or a nutritional supplement (Viusid) [18] with lifestyle modifications to induce weight loss in patients with NASH, and they generally reported improvement in histological features associated with NASH except for fibrosis.

Based on the available data, the recent multisociety practice guidelines recommend a goal of at least 3–5 % weight loss for reducing hepatic steatosis and a larger reduction of weight up to 10 % of baseline weight to improve hepatic necroinflammation in patients with NASH [22]. An interesting study from Cuba was just published, and it reported the findings from a prospective inception cohort study of weight loss through lifestyle modification in 293 patients with histologically confirmed NASH [23]. Two hundred and sixty-one patients underwent a follow-up liver biopsy at 52 weeks. At week 52, 30 % of the participants achieved a weight loss ≥ 5 %, and there was a strong stepwise relationship between the magnitude of weight loss and the degree of histological improvement. Notably, individuals achieving ≥10 % weight loss exhibited dramatic histological improvement including improvement in fibrosis (Table 16.1).

Adequately powered studies addressing the role of macronutrient composition in managing NAFLD are sparse [19, 24]. A recent report suggested a greater reduction in hepatic triglycerides as measured by magnetic resonance spectroscopy (−55 ± 14 % versus −28 ± 23 %) following 2 weeks of carbohydrate-restricted diet as compared to 2 weeks for calorie-restricted diet despite a similar degree of weight loss with both diets (−4.6 ± 1.5 kg versus −4.0 ± 1.5 kg) [10]. Whether these changes in hepatic fat content are sustainable over time with these different diets is currently unknown. There are however data on the long-term sustainability of weight loss with different diets. A large randomized trial using four different hypocaloric diets with emphasis on different macronutrients showed no significant differences between diets including those with higher or lower carbohydrate portions after 2 years of dietary modification [25]. This sup-

Table 16.1 Improvement of histological outcomes across different categories of weight loss at the end of treatment

Variables	Overall $n=293$	WL < 5 % $n=205$	WL = 5–6.99 % $n=34$	WL = 7–9.99 % $n=25$	WL ≥ 10 % $n=29$	*P* value**
Weight loss (%)	3.8 ± 2.7	1.78 ± 0.16	5.86 ± 0.09	8.16 ± 0.22	13.04 ± 6.6	–
Resolution of steatohepatitis[a]	72 (25)	21 (10)	9 (26)	16 (64)	26 (90)	<0.01
NAS improvement[b]	138 (47)	66 (32)	21 (62)	22 (88)	29 (100)	<0.001
– Change in NAS from baseline	−1.58 ± 0.27	−0.89 ± 0.13	−1.94 ± 0.36	−3.84 ± 0.29	−4.10 ± 0.23	<0.001
Steatosis improvement	142 (48)	72 (35)	22 (65)	19 (76)	29 (100)	<0.001
– Change from baseline	−0.63 ±0.10	−0.36 ± 0.07	−1 ± 0.13	−1.40 ± 0.19	−1.69 ± 0.12	<0.001
Lob. Inflammation improvement	147 (50)	72 (35)	24 (71)	22 (88)	29 (100)	<0.001
– Change from baseline	−0.49 ± 0.15	−0.29 ± 0.05	−0.53 ± 0.22	−1.32 ± 0.09	−1.21 ± 0.11	<0.001
Ballooning improvement	115 (39)	54 (26)	14 (41)	21 (84)	26 (90)	<0.001
– Change from baseline	−0.45 ± 0.17	−0.24 ± 0.04	−0.41 ± 0.13	−1.12 ± 0.13	−1.34 ± 0.08	<0.001
Fibrosis status[c]						<0.01
– Regression	56 (19)	33 (16)	6 (18)	4 (16)	13 (45)	
– Stabilized	191 (65)	129 (63)	25 (74)	21 (84)	16 (55)	
– Worsened	46 (16)	43 (21)	3 (8)	0 (0)	0 (0)	
– Change from baseline	−0.01 ± 0.02	0.09 ± 0.07	−0.02 ± 0.03	−0.17 ± 0.12	−0.86 ± 0.20	<0.001**
Portal inflammation improvement	44 (15)	27 (13)	3 (9)	5 (20)	9 (31)	0.049
– Change from baseline	0.02 ± 0.02	0.06 ± 0.01	0.09 ± 0.03	−0.07 ± 0.01	−0.31 ± 0.08	<0.01**
NAS						<0.001
NAS ≤ 2	119 (41)	48 (23)	20 (59)	22 (88)	29 (100)	
NAS 3–4	79 (27)	74 (36)	2 (6)	3 (12)	0 (0)	
NAS ≥ 5	95 (32)	83 (41)	12 (35)	0 (0)	0 (0)	

Reproduced with permission from [23]

Abbreviations: *WL* weight loss, *NASH* nonalcoholic steatohepatitis. Qualitative data expressed as *n* (%) and quantitative data as mean ± SD

[a]Resolution of steatohepatitis was defined as absence of the histological features of definite steatohepatitis, which required lack of hepatocellular ballooning with no fibrosis impairment

[b]NAFLD activity score (NAS) improvement indicates a reduction by at least two points in the NAS in comparison to baseline values with no fibrosis impairment

[c]Patients were classified as regression (decrease of at least 1 point in the fibrosis score), stabilized (no changes in the fibrosis score), and worsened (increase of at least 1 point in the fibrosis score)

[**]Significant difference (P < .01) between WL >10% as compared with WL between 7% and 10%, adjusted by Bonferroni test.

ports the current multi-society practice guidelines recommendation of hypocaloric diet to achieve weight loss without specific emphasis on any macronutrient [22].

While increased dietary intake of fructose particularly in corn syrup has been suggested to contribute to the increased risk of NAFLD and even more severe NAFLD histology [26–28], more recent reports have challenged this proposition. Although increased consumption of carbohydrates including fructose correlated with obesity risk, fat and total energy intake had more

influence on intrahepatic triglycerides content [29, 30].

The benefit of exercise as part of the recommended lifestyle modification for NAFLD patients has been demonstrated in several studies [5, 7, 11, 13, 14, 16, 31–33]. The degree of physical fitness correlates with the risk of NAFLD, with lower fitness strongly correlating with presence of NAFLD and increased fitness correlating with resolution of fatty liver by magnetic resonance spectroscopy [32]. Furthermore, aerobic and resistance exercise training may improve insulin sensitivity and hepatic steatosis independent of weight loss [11, 14, 31, 34].

The limited long-term durability of weight loss achieved through lifestyle modification represents a practical limitation to this approach as an effective strategy to manage NAFLD. Most of the weight loss is seen in the first 6 months of these interventions and may reach up to 10 % of initial body weight. One study randomized 811 subjects to 4 types of reduced caloric diets with variation in carbohydrate, protein, and fat content for 2 years [25]. At 6 months, the average weight loss was 6 kg, and many subjects began regaining weight after the first year. By 2 years, the average weight loss was 4 kg, and only 15 % of the subjects were able to lose more than 10 % of their baseline weight. Their macronutrient composition did not affect the degree of weight loss among the different study groups.

A systematic review evaluated 80 randomized trials of different weight loss modalities (total of 24,698 subjects, 68 % completed the planned studies) that had a minimum of 1 year follow-up [35]. Weight loss was achieved through diet or exercise alone, diet and exercise, meal replacements, very-low-energy diets, medications (orlistat or sibutramine), or advice alone. After an initial average weight loss of 5–8.5 kg, weight plateaued at 6 months. Only an average of 3–6 kg (3–6 %) of the weight loss was maintained at 4 years (Fig. 16.1). No significant weight loss was achieved in subjects who received advice or exercise alone.

On the other hand, morbidly obese patients who have a high prevalence of NAFLD experience sustainable weight loss reaching up to 25 %

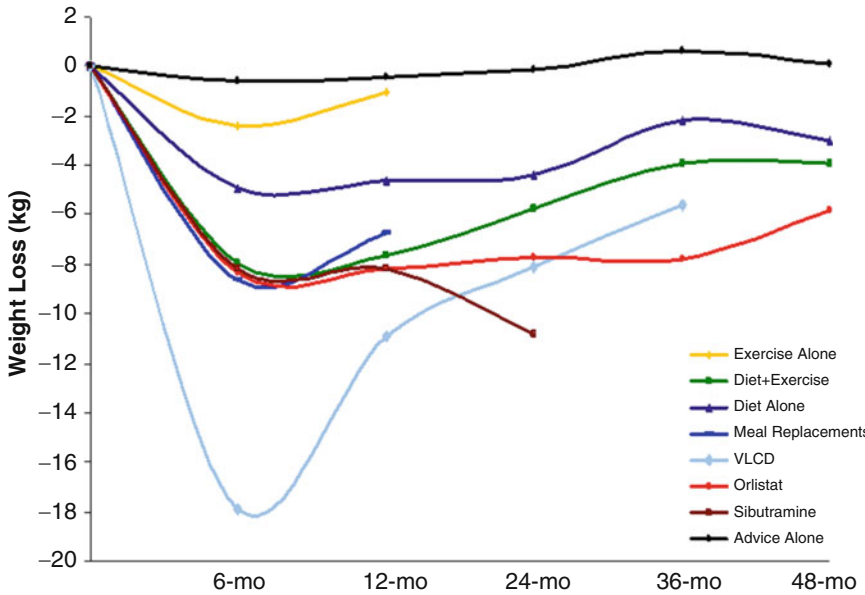

Fig. 16.1 Long-term weight loss with various types of nonsurgical methods. Courtesy Dr. Marion Franz. Reproduced Franz MJ, VanWormer JJ, Crain AL, Boucher JL, Histon T, Caplan W, et al. Weight-loss outcomes: a systematic review and meta-analysis of weight-loss clinical trials with a minimum 1-year follow-up. J Am Diet Assoc. 2007;107(10):1755–67 [35], with permission of the American Dietetic Association

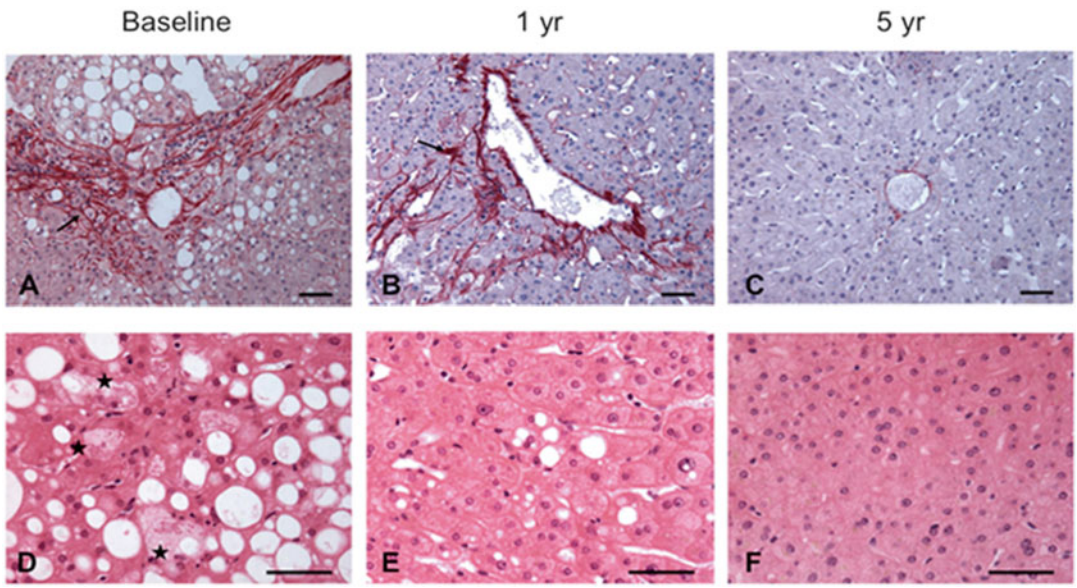

Fig. 16.2 Resolution of both steatohepatitis and significant fibrosis following Roux-en-Y gastric bypass surgery. Courtesy Dr. Francois Pattou. Reproduced from Caiazzo R, Lassailly G, Leteurtre E, Baud G, Verkindt H, Raverdy V, et al. Roux-en-Y gastric bypass versus adjustable gastric banding to reduce nonalcoholic fatty liver disease: a 5-year controlled longitudinal study. Ann Surg. 2014;260(5):893–9 [36], with permission of Wolters Kluwer Health

of initial body weight at 5 years following bariatric surgery [36]. A recent systematic review of bariatric surgery studies with at least 2 years of follow-up showed that both gastric bypass and sleeve gastroplasty consistently resulted in a minimum of >50 % excess weight loss, an outcome observed only in 31 % of gastric banding studies [37]. All types of bariatric procedures resulted in improvement in associated comorbidities including type 2 diabetes, dyslipidemia, and hypertension [37]. The effects of bariatric surgery on NAFLD have been reported in numerous studies and summarized in recent meta-analysis and systematic review [36, 38–46]. The majority of patients with NAFLD experience improvement or resolution of steatosis and steatohepatitis (Fig. 16.2). Improvement in fibrosis including resolution of cirrhosis has been reported, but some studies reported an increase in mild fibrosis in 7–40 % of the patients [41, 44, 47–49].

A careful look at the five studies reporting worsening fibrosis (Table 16.2) reveals that three of these studies used wedge biopsies which tend to be associated with increase in peripheral fibrosis and frequently lead to overestimation of fibrosis [47–49]. The remaining two studies came from the same group and reported an increase in fibrosis at 1 and 5 years following bariatric surgery [41, 44]. Liver histology was evaluated with core liver biopsies, and a considerable proportion of patients (42 %) underwent biliointestinal bypass in the first study [41], a procedure known to be associated with increased risk for development of hepatic fibrosis [50]. In the second study from this group, biliointestinal bypass was performed in 23 % of the patients [44]. About 80 % of patients had fibrosis regression, whereas 20 % experienced fibrosis progression at 5 years following bariatric surgery. In this cohort, >90 % of patients with fibrosis progression went from stage 0 to 1; and in the total cohort with follow-up biopsies, 96 % had fibrosis stage ≤1, and 0.5 % had stage 3. These patients remained morbidly obese at 5 years following surgery (BMI 40.5 ± 8.3 kg). Only one patient who underwent biliointestinal bypass progressed to cirrhosis in the setting of alcohol abuse. On multivariate analysis, only underlying fibrosis but not BMI,

Table 16.2 Summary of bariatric surgery studies reporting increase in hepatic fibrosis

Author	*N*	Baseline BMI (kg/m^2)	Procedure	Baseline fibrosis	Number with repeat biopsy	Interval to repeat histology (months)	Method of liver biopsy	Fibrosis evolution	Comments
Kral et al. [48]	689	47±9	Biliopancreatic diversion	14 (2 %) with baseline cirrhosis	104	41±25	Wedge	Increased 40 %, decreased 27 % Stable 33 %	Severe fibrosis decreased in 28, whereas mild fibrosis appeared in 42 11 patients with cirrhosis had decreased fibrosis by 2 stages 7 patients had disappearance and 2 regression of nodules and fibrous bridging
Stratopoulos et al. [49]	51	52.8±1	Vertical band gastroplasty	F0 6 % F1 33 % F2 45 % F3 6 % F4 0 %	51	18±9	Wedge	F0 14 % F1 49 % F2 27 % F3 10 % F4 0 %	16 subjects had a third liver biopsy 17±6 months after the second biopsy showing significant reduction fibrosis
Csendes et al. [47]	557	44.3	Roux-en-Y gastric bypass	Only 1 with any fibrosis and had cirrhosis	16	17	Wedge	1 with new stage 1 fibrosis Stable cirrhotic but with resolution of NASH	
Mathurin et al. [41]	171	49±8	Gastric band (58 %) Biliointestinal bypass (42 %)	F0 81 % F1 10 % F2 2 % F3 1 % F4 6 %	121	12	Core needle	Mean fibrosis score, increased from 0.14±0.39 to 0.38±0.64 % patients without significant fibrosis <2 was not significantly different between the preoperative and the postoperative period: (98.6 % vs. 95 %)	Increase in mean fibrosis score not clinically relevant Bariatric surgery not done in 14 patients with suspected cirrhosis
Mathurin et al. [44]	376	50±8	Gastric band (56 %) Biliointestinal bypass (23 %) Gastric bypass (21 %)	F0 77 % F1 18.5 % F2 4 % F3 0.5 % F4 0 %	211	60	Core needle	Fibrosis regression 80 % progression 20 % Fibrosis progression mainly from stage 0 to1 In the total cohort with follow-up biopsies, 96 % had fibrosis stage ≤1 and 0.5 % had stage 3	Patients with fibrosis progression remained morbidly obese at 5 years following surgery (BMI 40.5±8.3 kg) One patient who underwent biliointestinal bypass progressed to cirrhosis in the setting of alcohol abuse

steatosis, inflammation, or ballooning influenced the progression. In this study, the investigators reported no significant differences in fibrosis progression between the gastric band, biliointestinal, and gastric bypass groups between baseline and 5 years. However, the investigators just published another follow-up report detailing their 5-year follow-up of their bariatric cohort [36], in which they declared that their center abandoned biliointestinal bypass due to concerns about association with liver fibrosis and replaced it with adjustable gastric band, Roux-en-Y gastric bypass, or sleeve gastrectomy. In this updated report, 13 patients had bridging fibrosis at baseline, which regressed in six and disappeared in two patients. There was no report of worsening fibrosis in this cohort without biliointestinal bypass from the same center. Roux-en-Y gastric bypass resulted in more weight loss and improvement in NAFLD histology in this report although patients were not randomized and the type of surgery was chosen by patients. Patients who received the adjustable gastric band had lower BMI, HOMA, and NAS scores at baseline. Based on these data, there is no convincing evidence that weight loss induced by Roux-en-Y gastric bypass, gastric band, or sleeve gastrectomy is associated with progression of hepatic fibrosis.

Bariatric surgery can therefore be an option to NAFLD patients with morbid obesity [22]. There is however paucity of data about the safety and optimal type of bariatric surgery for patients with NASH-related cirrhosis, with the few case series including patients with cirrhosis reporting improvement in NASH histology and regression of cirrhosis in many patients [47, 48].

Vitamin E

It has long been contended that oxidative stress contributes to the pathogenesis of NASH [51–56]. Based on its known function as an antioxidant [57], vitamin E has been used alone or with other compounds in multiple trials to treat NASH or NAFLD [1, 58–66]. Varying dosages ranging from 100 to 1200 IU/day have been used with reported beneficial effects on liver enzymes, steatosis, inflammation, ballooning, and hepatic fibrosis. The duration of vitamin E monotherapy in these trials ranged from 4 to 96 weeks [65, 67]. Vitamin E effects on ALT could be seen in as early as 4 weeks [65] and on histology in 6 months [60, 61]. In one of the largest randomized trials in NASH to date, the PIVENS [63], vitamin E was given at dose of 800 IU/day for 96 weeks and compared to pioglitazone or placebo. Vitamin E and pioglitazone improved ALT, steatosis, lobular inflammation, and ballooning and resulted in resolution of NASH in a significant number of patients. There was no improvement in fibrosis. Vitamin E, however, was not associated with weight gain as pioglitazone. This trial excluded patients with NASH if they had diabetes or cirrhosis. In the TONIC clinical trial [66], which compared the efficacy of vitamin E to that of metformin in children with biopsy-proven NAFLD, significant improvement in ballooning grade and NAFLD activity score was noted only with vitamin E. Other trials with vitamin E monotherapy were small, and either did not include diabetics or included a small subgroup of diabetics.

A meta-analysis by Miller et al. raised concerns about an increase in all-cause mortality with vitamin E use [68]. Although other meta-analyses confirmed this finding [69, 70], these results were contested by other analyses which did not show an association but raised the possibility of underlying patients' condition as a possible cause of increased mortality associated with vitamin E intake [71, 72]. More recently, an increased rate of prostate cancer was reported in a trial with vitamin E administered to healthy men [73]. The multi-society practice guidelines recommend considering vitamin E at 800 IU daily as a first-line therapy for patients with histologically confirmed NASH without cirrhosis or type 2 diabetes [22].

Thiazolidinediones

The peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonists, rosiglitazone and pioglitazone, improve insulin sensitivity by decreasing glucose production and increasing its

utilization in the muscle, adipose, and liver tissue. Thiazolidinediones (TZDs) induce favorable effects on production of adiponectin, resistin, interleukin-6, and tumor necrosis-alpha and reduce circulating free fatty acids, thus modulating inflammation and atherosclerosis [74–77]. Pioglitazone also has a PPAR-α effect which mediate hepatic fatty acid oxidation and inflammation [78].

Several small studies evaluated the effects of rosiglitazone on NAFLD [79–84]. Initial reports suggested improvement in all NASH histological features. However, the FLIRT2 trial examined the long-term effects of extending the use of rosiglitazone for 2 additional years in subjects who already participated in an initial 1 year placebo-rosiglitazone trial [82]. The prolonged use of rosiglitazone in this trial was only associated with continued improvement in ALT, insulin sensitivity, and steatosis, but not hepatic necroinflammation or fibrosis.

The beneficial effects of pioglitazone on NAFLD have also been shown in several clinical trials [60, 63, 85–87]. In the PIVENS clinical trial, pioglitazone resulted in NASH resolution in 34 % of the subjects compared to 43 % of subjects on vitamin E and 19 % for the placebo group. Pioglitazone significantly improved steatosis and necroinflammation, but not fibrosis in nondiabetic, non-cirrhotic subjects with NASH in this trial [63]. A recent meta-analysis of data from this study in addition to two prior trials of pioglitazone in NASH [86, 87] confirmed the improvement in steatosis and necroinflammation but also showed improvement in fibrosis in the pooled data (Fig. 16.3) [88].

Except for increased weight (4.7 kg over the study 96-week period), pioglitazone was well tolerated and did not have higher adverse events compared to placebo in this trial.

However, the metabolic and histological improvements seen with pioglitazone use reverse

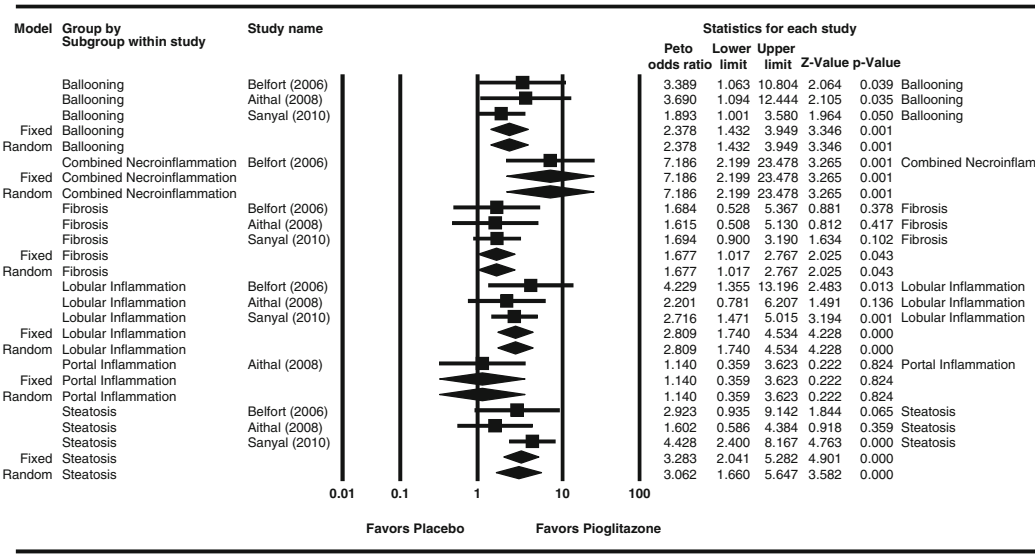

Fig. 16.3 Effects of pioglitazone on liver histological features in nonalcoholic steatohepatitis. Courtesy Dr Rohit Loomba. Reproduced from Boettcher E, Csako G, Pucino F, Wesley R, Loomba R. Meta-analysis: pioglitazone improves liver histology and fibrosis in patients with nonalcoholic steatohepatitis. Aliment Pharmacol Ther. 2012;35(1):66–75 [88], with permission of John Wiley and Sons

upon its discontinuation. In a follow-up study of 21 patients after 48 weeks of pioglitazone therapy, 13 subjects were followed for an additional 48 weeks, and 9 subjects had repeated liver biopsy [89]. Rebound increases in ALT and HOMA, decrease in adiponectin level, and worsening steatosis and inflammation were observed. This study not unexpectedly shows that long-term use of pioglitazone is necessary to maintain the associated metabolic and hepatic benefits.

While the majority of patients with NASH in the TZD trials were nondiabetics, there is continuing controversy about the TZDs long-term safety for treating type 2 diabetes. Reports of increased incidence of bone fractures, congestive heart failure, and bladder cancer have raised concern about TZDs safety [90, 91]. The cardiovascular profile for pioglitazone may be better than that of rosiglitazone as it was associated with lower risk for cardiovascular events, including congestive heart failure and myocardial infarction, in a recent meta-analysis [92]. These different cardiovascular effects were postulated to be related to the different effects pioglitazone exerts on metabolic genes in addition to observed improvements in triglycerides and high-density lipoprotein cholesterol levels compared to rosiglitazone.

The current multi-society practice guidelines recommend that pioglitazone may be used with caution in nondiabetic patients with biopsy-proven NASH [22].

Obeticholic Acid

Since the discovery of the bile acid nuclear receptor farnesoid X receptor (FXR) [93, 94], there has been considerable progress in the understanding of its biological functions. FXR plays an essential role in regulating bile acids synthesis and transport, lipid and glucose homeostasis, and hepatic inflammation [95, 96]. Obeticholic acid (OCA) is a semisynthetic derivative of the primary human bile acid chenodeoxycholic acid, the natural agonist of the FXR. Based on its potent and selective FXR agonist effects, OCA has been tested as a potential therapeutic agent for NASH in two clinical studies. The initial study was a pilot human trial of short duration consisting of 64 patients with nonalcoholic fatty liver disease and type 2 diabetes mellitus [97]. Patients were randomized to receive placebo, 25 mg OCA, or 50 mg OCA once daily for 6 weeks. The primary end point of the study was improvement in insulin sensitivity as measured by the hyperinsulinemic-euglycemic clamp technique. Changes in liver enzyme levels were among several measured secondary end points. Compared to placebo, OCA improved insulin sensitivity, ALT, and Enhanced Liver Fibrosis test, a validated serum marker of fibrosis. Importantly, treatment was associated with weight loss. Despite remaining within the normal range, there was a mild increase in alkaline phosphatase levels in subjects receiving OCA compared to placebo.

This study was followed by a large multicenter randomized clinical trial (FLINT) of oral OCA in patients with NASH without cirrhosis [98]. In total, 283 patients with biopsy-proven NASH and NAFLD activity score (NAS) of ≥4 were randomized to receive OCA 25 mg orally daily or matching placebo for 72 weeks. Nearly 50 % of the subjects had type 2 diabetes. The primary outcome of this study was improvement in liver histology defined as a decrease in NAS by at least 2 points without worsening of fibrosis from baseline to the end of treatment. Due to its vanguard study design, after a planned interim analysis showed significant beneficial effects of OCA on histology, treatment was ended early in the last 64 subjects and a follow-up liver biopsy was not obtained for them. A greater number of participants receiving OCA met the primary study outcome on OCA as compared to placebo (45 % versus 21 %, relative risk 1.9, 95 % CI 1.3–2.8; $p=0.0002$). There was significant improvement noted in steatosis, lobular inflammation, ballooning, and fibrosis with OCA (Table 16.3). Although resolution of definite NASH was observed more frequently with OCA than placebo (22 versus 13 %), this did not reach statistical significance ($p=0.08$).

Table 16.3 Effects of 72 weeks of obeticholic acid therapy on liver histology in patients with NASH

	Obeticholic acid	Placebo	Relative risk or change Obeticholic acid vs. placebo (95 % CI)	*P*-value
Primary outcome				
No. of patients at risk[a]	110	109		
Patients with improvement—*n* (%)	50 (45.5)	23 (21.1)	1.9 (1.3, 2.8)	0.0002
Changes from baseline in histologic features				
No. of patients with biopsy specimens at baseline and 72 weeks	102	98		
Resolution of definite nonalcoholic steatohepatitis—*n* (%)	22 (21.6)	13 (13.3)	1.5 (0.9, 2.6)	0.08
Fibrosis[b]				
Patients with improvement—*n* (%)	36 (35.3)	19 (19.4)	1.8 (1.1, 2.7)	0.004
Change in score—mean ± SD	−0.2 ± 1.0	0.1 ± 0.9	−0.3 (−0.6 to −0.1)	0.01
Total NAFLD activity score				
Change in score—mean ± SD	−1.7 ± 1.8	−0.7 ± 1.8	−0.9 (−1.3 to −0.5)	<0.0001
Hepatocellular ballooning				
Patients with improvement—*n* (%)	47 (46.1)	30 (30.6)	1.5 (1.0, 2.1)	0.03
Change in score—mean ± SD	−0.5 ± 0.9	−0.2 ± 0.9	−0.2 (−0.5 to 0.0)	0.03
Steatosis				
Patients with improvement—*n* (%)	62 (60.8)	37 (37.8)	1.7 (1.2, 2.3)	0.001
Change in score—mean ± SD	−0.8 ± 1.0	−0.4 ± 0.8	−0.4 (−0.6 to −0.2)	0.00041
Lobular inflammation				
Patients with improvement—*n* (%)	54 (52.9)	34 (34.7)	1.6 (1.1, 2.2)	0.006
Change in score—mean ± SD	−0.5 ± 0.8	−0.2 ± 0.9	−0.3 (−0.5 to −0.1)	0.0006
Portal inflammation				
Patients with improvement—*n* (%)	12 (11.8)	13 (13.3)	1.0 (0.6, 1.7)	0.90
Change in score—mean ± SD	0.2 ± 0.7	0.2 ± 0.7	0.0 (−0.1 to 0.2)	0.59

Reproduced with permission from Neuschwander-Tetri et al. [98]

[a] Number of randomly assigned patients with observed or expected week 72 visit before protocol modifi ed on Jan 6, 2014, to eliminate week 72 biopsy

[b] Fibrosis was assessed on a scale of 0–4, with higher scores showing more severe fibrosis

Pruritus occurred more commonly with OCA (23 % versus 6 %). OCA therapy was associated with a decrease HDL and increase in total and LDL cholesterol at 12 weeks of therapy. These changes attenuated with therapy and resolved after discontinuation of OCA. Similar to the earlier OCA study, there was mild increase in alkaline phosphatase in the OCA group. There was an average weight loss of 2.3 kg with OCA compared to no weight loss in the placebo group. Five severe or life-threatening events that were deemed related to OCA including three events of severe pruritus, one of hyperglycemia, and one of possible cerebral ischemia. There were two deaths in subjects receiving OCA and were deemed not related to the study drugs (one from myocardial ischemia and another from sepsis and heart failure).

The favorable effects of OCA on liver histology are encouraging. Additional studies to validate the findings and provide longer-term follow-up will be needed to confirm efficacy and define the safety profile of this agent.

Liver Transplantation

Liver transplantation is a therapeutic option for patients with NASH cirrhosis who develop liver failure or hepatocellular carcinoma. Indeed, there has been a considerable increase in liver transplan-

tation for NASH over the past decades. NASH is currently the second most common indication for liver transplantation in the USA and is projected to become the leading indication in the next one to two decades [99–102]. Patients with NASH cirrhosis who are listed for liver transplantation are usually older and have higher BMI and lower incidence of HCC than those listed for other indications [99, 103]. Despite a more complex transplantation course marked by increased intraoperative blood loss, longer operative times, and posttransplant length of stay [101], the 1- and 3-year posttransplant survival for patients transplanted for NASH cirrhosis are excellent (average 85 % and 78 %, respectively); they are at least comparable to that autoimmune disease and better than hepatocellular carcinoma, hepatitis C virus, alcoholic liver disease, acute hepatic necrosis, and hemochromatosis [99–101, 104]. Although NAFLD and NASH recur following transplantation in up to 40 % of the patients, graft and patient survival are not affected at least in the short to midterm [101, 105–107]. Meticulous selection of candidates with NASH cirrhosis for liver transplantation is necessary for optimal outcomes posttransplantation. Given the high prevalence of cardiovascular disease in patients with NAFLD, potential candidates should undergo a thorough pre-transplant cardiac evaluation to diagnose and treat underlying cardiac disease [108]. Optimization of the metabolic syndrome and obesity management is also an important aspect of the care in the pre- and posttransplantation setting [109].

Other Agents

Metformin

Metformin is a commonly used hypoglycemic agent. It inhibits hepatic gluconeogenesis, enhances peripheral tissue utilization of glucose, reduces circulating free fatty acids, and decreases food intake and body weight, changes that are collectively associated with improved insulin sensitivity [110–112].

Metformin has been tested as a treatment for NAFLD or NASH in several trials. Initial small studies reported improvement in ALT, steatosis by imaging, and liver histology with metformin [113–119]. In the largest clinical trial in adults [113], 110 nondiabetic Italian subjects with NAFLD (mean age 43 years, BMI 28.8 kg/m^2) were randomized to receive metformin (2 g/day; $n=55$) versus vitamin E (800 IU/day; $n=28$) versus dietary intervention to reduce weight ($n=27$) for 12 months. ALT improved in all cases. In the 17 subjects who received metformin and agreed to repeat liver biopsy, significant improvement in hepatic steatosis, necroinflammation, and fibrosis was observed. However, metformin effects on liver histology could not be reproduced in several subsequent studies [66, 120–122]. In the largest randomized trial testing the effects of metformin to date, the TONIC clinical trial [66], 173 children (aged 8–17 years) with biopsy-confirmed NAFLD were randomized to receive vitamin E (800 IU/day, $n=58$), metformin (1000 mg/day, $n=57$), or placebo ($n=58$) for 96 weeks. Neither metformin nor vitamin E showed significant difference compared to placebo in achieving sustainable decrease in ALT. Reduction in ballooning grade and NAFLD activity score, and increase in proportion of patients with NASH resolution with both metformin and vitamin E, reached statistical significance only with vitamin E, but not metformin therapy. There was no significant improvement in the other NAFLD histological features with either therapy compared to placebo. A meta-analysis that pooled the results from four studies with metformin found no effect for metformin on liver histology in patients with NAFLD [123]. Based on this data, the multi-society practice guidelines do not recommend metformin for the treatment of liver disease in patients with NASH [22].

Ursodeoxycholic Acid

Ursodeoxycholic acid (UDCA) has many attractive putative mechanisms of actions that prompted testing it as a potential therapy for NAFLD. In addition to altering the bile acid pool, UDCA has choleretic, anti-inflammatory and anti-apoptotic effects and may modulate immune response and mitochondrial integrity [124].

Earlier studies reported improvement of ALT and steatosis in patients with NAFLD with UDCA at a daily dose of 12–15 mg/kg alone or when combined with vitamin E [62, 64, 125]. Another study using high-dose UDCA (28–35 mg/kg/day for 12 months) in 126 subjects with biopsy-proven NASH showed reduction in ALT together with improvement in serum markers of insulin resistance and hepatic fibrosis [126]. However, two large randomized studies in patients with biopsy-confirmed NASH at baseline and with histological end points failed to show significant effects for UDCA on NASH histology with low-dose (13–15 mg/kg/day for 2 years, $n=166$) and high-dose (23–28 mg/kg/day for 1.5 years, $n=185$) UDCA [127, 128]. This data does not support the use of UDCA for treatment of NAFLD or NASH.

Statins

The hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase) inhibitors, also known as statins, are widely used for the treatment of dyslipidemia and primary and secondary prevention of cardiovascular disease [129, 130]. Several small reports suggest that these agents are safe when used in patients with NAFLD and may result in decreasing ALT levels and hepatic steatosis on imaging [131–135]. Two small studies suggested an improvement in histological features other than fibrosis, but no histological changes were noted in a third study [136–138]. Based on this inconclusive data and lack of adequately designed randomized trials, statins cannot be recommended as treatment for NASH.

However, in patients with NAFLD or NASH, statins can be safely used to treat dyslipidemia as demonstrated by the above studies and data from larger studies specifically looking at their safety in the setting of NAFLD and other liver diseases [139–141]. In a large study that evaluated the safety of statins in dyslipidemic patients with underlying liver disease, there was no significant difference in the incidence of elevated liver tests of varying severity after 6 months of use between subjects with and without elevated baseline liver enzymes [140]. In a randomized controlled trial of high-dose pravastatin in patients with compensated chronic liver disease and dyslipidemia [141], the incidence of ALT elevation to more than twice the upper limit of normal at the end of 36-week trial was lower in subjects who received pravastatin compared to placebo (7.5 % vs. 12.5 %), although this did not reach statistical significance ($p=0.13$). Another large study (the GREACE study) showed that in patients with underlying coronary artery disease and elevated transaminases presumably due to NAFLD, those who received statin (88 % received atorvastatin) experienced an improvement in their liver tests compared to those who did not [139]. More importantly, they suffered lower number of cardiovascular events compared to those with elevated liver tests who did not receive a statin (10 % vs. 30 %, $p<0.0001$). Less than 1 % of study subjects discontinued statin due to elevation in transaminase to more than three-times the upper limit of normal per study protocol. All together, these data suggest that there is no evidence for increased severe hepatotoxicity in patients with NAFLD who receive statins.

Fibrates

Fibrates are commonly used to treat hypertriglyceridemia [77]. By activating PPAR-α, fibrates increase hepatic fatty acid oxidation and reduce hepatic triglyceride synthesis and VLDL production and export [142]. In patients with the metabolic syndrome, fibrates reduce plasma triglyceride, C-reactive protein, and interleukin-6 without affecting hepatic or peripheral insulin sensitivity or circulating free fatty acids [143, 144].

There are a few small studies exploring the effects of fibrates on NAFLD with conflicting fibrates effects on ALT [125, 145, 146]. One study showed no effect of 8 weeks therapy with fenofibrate on hepatic fat content despite decreasing plasma triglycerides and VLDL [147]. Two studies reported fibrates effect on liver histology in NAFLD with one showing improvement only in ballooning, while the other reported no improvement in histology [125, 146]. Based on current available data, fibrates cannot be recommended to treat NASH.

Long-Chain Polyunsaturated Fatty Acids

Long-chain polyunsaturated fatty acids (LC-PUFA) in the n-3 (omega-3: ω-3) series including docosahexaenoic acid (DHA; C22:6n-3) and eicosapentaenoic acid (EPA; C20:5n-3) are abundant in fish and fish oil supplements and exert several beneficial biological effects. In addition to lowering triglycerides and increasing HDL, LC-PUFA increase circulating adiponectin, improve insulin sensitivity, and reduce body weight, adipose tissue inflammation, endothelial dysfunction, and coronary artery disease risk [148–150]. These effects are highly desirable in patients with NAFLD in whom cardiovascular disease is the leading cause of death. Based on analysis of the National Health and Nutrition Examination Survey (NHANES) 2003–2008 data, a majority of US adults do not consume the recommended daily amount of LC-PUFA [151]. There is also data to suggest the LC-PUFA desaturation is altered in NASH with imbalance between the pro-inflammatory (ω-6 pathway) and the anti-inflammatory (ω-3) pathways [152].

LC-PUFA have been tested as treatment of NAFLD in several studies with reports of improved ALT and hepatic steatosis by imaging [153–158]. These studies were limited either by small size, non-biopsy diagnosis of NAFLD, or design issues. A meta-analysis of data pooled from these studies showed significant heterogeneity among studies but yielded consistent effect for LC-PUFA on improving hepatic steatosis [159]. The pooled data could not confirm improvement in transaminases with this therapy. A recent study randomized 37 diabetic patients with NASH to receive a combination of EPA+DHA (4 g/day) or placebo containing corn oil for 48 weeks [160]. There was no change in liver enzymes or histology with LC-PUFA in this study. In a randomized placebo-controlled study of 103 subjects with NAFLD (the WELCOME study), 15–18 months treatment with DHA+EPA (4 g/day) did not result in significant decrease in hepatic fat content or serum markers of fibrosis [161]. The lack of effect was attributed to compliance in the treatment arm and contamination with DHA/EPA in the placebo group. There was a significant correlation noted between the increase in erythrocytes enrichment with DHA and decreasing hepatic fat content. In a large multicenter trial of EPA, 243 subjects with biopsy-proven NASH were randomized to receive placebo, EPA 1800 mg/day, or EPA 2700 mg/day for 12 months. EPA therapy had no effects on ALT, liver histology, or serum levels of keratin-18, hyaluronic acid, C-reactive protein, or insulin resistance [162]. Finally, in a recent study in 41 patients with NASH but without cirrhosis, subjects were randomized to receive 3 g of n-3 fish oil or placebo for 12 months [163]. Treatment with fish oil had no significant effects on NASH histological lesions compared to placebo. Subgroup analysis of subjects who maintained or increased their weight during the study showed a nonsignificant trend for reduction in liver fat by morphometric and MRI quantifications in the treatment arm but not by the standard semiquantitative scoring of steatosis on liver biopsy. To date, no study has yet explored the efficacy of purified DHA alone as a potential therapy for NASH. This approach is attractive because experimental data suggest DHA may be more potent than EPA in suppressing hepatic lipogenesis, inflammation, oxidative stress, and fibrosis [164, 165].

Angiotensin Antagonists

There is evidence for a role of the renin-angiotensin system in regulating hepatic stellate cells and fibrogenesis. Activation of the angiotensin II receptor 1 results in stellate cells activation and proliferation, whereas blocking this receptor or angiotensin II results in stellate cell apoptosis and diminished hepatic fibrosis [166–168]. In a cross-sectional retrospective study, the use of angiotensin-converting enzyme inhibitors (ACEI) or angiotensin receptor blockers (ARB) in patients with NAFLD and hypertension was associated with milder degree of fibrosis and ballooning [169]. One study randomized 150 patients with NAFLD to receive either

losartan or amlodipine for 6 months followed by simvastatin [170]. Losartan resulted in reduction in hepatic steatosis, visceral adipose tissue, and insulin resistance compared to amlodipine, and these effects were further enhanced by adding simvastatin. In another study of 54 patients with NASH and hypertension, patients were randomized to receive one of two ARBs, valsartan or telmisartan for 20 months [171]. ALT, HOMA, and liver histology improved in both groups. Both drugs resulted in significant reduction in steatosis, but only telmisartan significantly improved lobular inflammation, ballooning, fibrosis, and the NAFLD activity score.

Pentoxifylline

Pentoxifylline is a nonselective phosphodiesterase inhibitor that has many putative functions including suppression of tumor necrosis factor-α, increasing hepatic glutathione, and reducing hepatic inflammation and oxidation of free fatty acids [172–174].

It has been tested as a treatment for NASH in a few small studies. Earlier reports suggested reduction of ALT [175, 176]. One small open-label study reported improvement in NAFLD activity score with pentoxifylline [177]. Similar results were shown in a randomized controlled trial of 55 patients with NASH, with improvement in steatosis, lobular inflammation, and fibrosis, but not ballooning after 1 year of pentoxifylline therapy [178]. However, another randomized study of 30 patients with NASH did not show significant improvement in NASH histology compared to placebo [179]. Additional large clinical trials are needed to test pentoxifylline's effects of NASH histology.

Emerging Therapies

Simtuzumab

Lysyl oxidases (LOX) are a family of extracellular matrix cross-linking enzymes involved in cross-linking collagen and elastin. Simtuzumab is a humanized monoclonal antibody to LOX like (LOXL) 2 [180]. Two clinical trials in patients with NASH and bridging fibrosis (NCT01672866) or cirrhosis (NCT01672879) are currently evaluating its safety and effects on hepatic venous pressure gradient, hepatic fibrosis, and overall and hepatic event-free survival.

GR-MD-02

Galectin 3 protein is important in hepatic fibrogenesis. GR-MD-02 is a complex carbohydrate galectin 3 inhibitor that improved fibrosis and portal hypertension in toxin-induced cirrhosis and resulted in regression of fibrosis in a murine model of NASH with fibrosis [181, 182]. The results of an early phase 1 trial have recently been presented [183] and demonstrated safety of this compound in patients with NASH and bridging fibrosis. This compound reduced serum markers of fibrosis (FibroTest® and Keratin-18) and inflammatory markers (tumor necrosis factor-alpha and interleukin-6 and 8) in studied subjects. A multicenter, phase 2 study of GR-MD-02 in patients with NASH cirrhosis is underway in the USA.

Exenatide

Exenatide is a synthetic glucagon-like peptide-1 (GLP-1) agonist. It exerts strong regulatory effect on postprandial insulin secretion and glucose metabolism [184].

There is early data from animal and human studies showing that exenatide reduces free fatty acid-induced endoplasmic reticulum stress and apoptosis [185] and results in improvement in hepatic steatosis in patients with type 2 diabetes by improving sensitivity to fibroblast growth factor 21 [186–188]. In a small open-label study, eight patients with diabetes and biopsy-proven NASH received exenatide for 28 week [189]. There was improvement in NASH histological lesions which resulted in reduction in the NAFLD activity score in five subjects. Fibrosis improved by one stage in three subjects and by two stages in one and worsened by one stage in one subject,

while it remained stable in the other three subjects. Large randomized controlled trials are needed to adequately assess exenatide effects on NASH histology.

Fibroblast Growth Factor 21

Fibroblast growth factor 21 (FGF21) is a member of the hormone-like FGF subfamily and is a potent regulator of metabolism and energy homeostasis [190, 191]. In murine models of NAFLD and NASH, FGF21 administration has been shown to improve hepatic steatosis, inflammation, and fibrosis [192–194]. In Ossabaw miniature swine with diet-induced NASH, FGF21 administration resulted in improvements in hepatic necroinflammation and fibrosis, insulin sensitivity, and postprandial lipidemia [195]. No clinical trials in humans have yet been undertaken to evaluate the effects of FGF21 on NASH.

Cenicriviroc

Cenicriviroc (CVC) is an oral inhibitor of the C-C chemokine receptors (CCR) 2 and 5, which are involved in macrophage recruitment to the liver. In a murine model of diet- and streptozotocin-induced NASH, there was reduction in fibrosis and necroinflammation in mice receiving this agent [196]. There is currently an ongoing phase 2 clinical trial (NCT02217475) that is evaluating the safety and efficacy of this agent in improving histology in patients with NASH and fibrosis but without cirrhosis.

GFT505

GFT505 is a dual PPAR α and δ agonist that improves insulin sensitivity and reduces serum triglycerides and low-density lipoprotein while increasing high-density lipoprotein levels with early animal studies suggesting favorable effects on NASH histology [197, 198]. In various models of liver injury, this agent improved hepatic steatosis, fibrosis, and inflammation [199]. In phase II clinical trials in subjects with the metabolic syndrome, GFT505 resulted in improvement in liver enzymes [199]. There is an ongoing phase 2 multicenter randomized clinical trial (NCT01694849) that is evaluating the effects of 52-week therapy with various doses of GFT505 on NASH histological lesions.

Cysteamine Bitartrate

Glutathione is an important endogenous antioxidant. Cysteamine, a glutathione precursor, is more effective at crossing cellular membranes than glutathione. It exerts an antioxidant effect that is protective against acetaminophen-induced liver injury in humans [200, 201]. In a recent pilot study, enteric-coated cysteamine was given for 24 weeks to 13 children with biopsy-proven NAFLD and elevated ALT [202]. In the 11 subjects who completed the study, 7 had normalization or >50 % reduction in ALT. There was also an increase in the mean serum adiponectin level and a decrease in keratin-18 levels. There is currently an ongoing clinical trial of in children with biopsy-proven NAFLD (NCT01529268) to evaluate the effects of three doses of cysteamine given for 52 weeks on NAFLD histology.

Management of Associated Metabolic Comorbidities and Environmental Risks

Patients with NAFLD have high prevalence of other components of the metabolic syndrome [203–205]. NAFLD is an independent risk factor for CVD and type 2 diabetes [206–210], while CVD is the leading cause of death in patients with NAFLD [206, 211, 212]. In addition to efforts aimed at improving the liver disease in patients with NASH, optimum control of the associated metabolic conditions and CVD risk factors such as smoking is important to optimize the survival of all patients with NAFLD, including those without NASH [213]. Weight reduction and excellent control of type 2 diabetes and dyslipidemia are recommended for all patients with

NAFLD [214, 215]. It is critical to not withhold statins from NAFLD patients who have indications for their use as there is ample evidence as summarized above for their safety in patients with liver disease including those with NAFLD [216]. Effective lifestyle modifications for patients with NAFLD are associated with improvement in the metabolic and CVD risks [214]. Weight loss induced through lifestyle modifications or bariatric surgery also results in dramatic improvement not only on NAFLD histology but also in associated metabolic conditions [42, 43, 214, 217]. There is emerging data to suggest that with NAFLD resolution, patients may be no longer at increased future risk for diabetes development [218].

Of the environmental risks, alcohol consumption warrants special attention. Alcohol effects on health and mortality have been the subject of continuing debate. In several studies, light to moderate alcohol consumption (less than two drinks or 40 g a day) increased serum HDL and reduced insulin resistance, triglycerides, and risk of type 2 diabetes, coronary artery disease, and mortality [219–224]. Light to moderate alcohol consumption has also been associated with lower prevalence of NAFLD and improved insulin sensitivity as suggested by lower HOMA scores [224–227]. A recent meta-analysis of pooled data from eight cohorts that included subjects with modest drinking (less than 40 g/day of alcohol) suggested a protective effect for modest drinking against NAFLD [228]. Another recent cross-sectional study from the NASH Clinical Research Network showed lower incidence of NASH in patients reporting modest alcohol consumption of <20 g/day [229]. In addition, modest drinking was associated with lower frequency of ballooning and fibrosis compared to no prior alcohol drinking history.

Despite these favorable reports, long-term safety of light to modest alcohol consumption in patients with or at risk for NAFLD is not well established. Short-term moderate alcohol consumption (16 g/day in women and 33 g/day for men) in healthy Swedish subjects for 90 days increased intrahepatic fat content by 1.1 % (range 0.2–3.9 %) [230]. Alcohol consumption exceeding one drink a day has also been associated with increased risk of alcoholic liver disease and death from cirrhosis and cancers of the upper gastrointestinal tract in large cohort studies [220, 231, 232]. Additionally, in a recent study of patients with NASH cirrhosis, any alcohol consumption including social drinking pattern was associated with significantly increased risk for hepatocellular carcinoma [233].

In summary, until prospective long-term studies assess the safety and benefits of alcohol use in patients with NAFLD, alcohol consumption cannot be recommended for these patients [22]. A comprehensive approach to identify and correct associated metabolic conditions and CVD risk factors is necessary to optimize the care and outcomes of patients with NAFLD.

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Nonalcoholic Fatty Liver Disease in Children

17

Hannah I. Awai, Kimberly P. Newton, and Jeffrey B. Schwimmer

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in children [1]. Diagnosis is made by the combination of clinical and histologic findings. Histologically, NAFLD ranges from isolated steatosis to steatosis with inflammation and varying degrees of fibrosis. There are notable histologic differences between childhood and adult NAFLD. It is possible that these differences are triggered by factors present prior to birth and in the early postnatal period. This chapter highlights epidemiology, pathophysiology, clinical features, diagnostic approaches, and emerging treatments unique to pediatric NAFLD.

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Diagnosis

NAFLD is not a singular diagnosis, but rather a clinical–pathological diagnosis that encompasses a broad spectrum of liver disease ranging from isolated steatosis to steatohepatitis, fibrosis, and cirrhosis [2]. The diagnosis of NAFLD requires histologic demonstration that 5 % or more of hepatocytes have macrovesicular steatosis. Other liver diseases or clinical conditions which may cause steatosis need to be excluded [3].

Histology

Adult NAFLD and pediatric NAFLD have some key histologic differences. Pediatric NAFLD is characterized by steatosis that is more severe, with greater portal inflammation and fibrosis, less Mallory's hyaline, a smaller degree of ballooning, and a smaller amount of perisinusoidal (zone 3) fibrosis [4]. Two types of histologic patterns have been described in NASH. Type 1, more common in adults, is characterized by steatosis, ballooning degeneration, and perisinusoidal fibrosis. Type 2,

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more common in non-Caucasian children, is characterized by steatosis, portal inflammation, and portal fibrosis [5]. Portal fibrosis was found to be more prominent in children with NAFLD in a study of 100 children where type 2 was the most predominant type present in 51 % of participants and was more commonly associated with advanced fibrosis [5]. Another study of 80 Korean children with NAFLD reported a higher prevalence of type 2 NASH in 44 % of participants, while type 1 was present in 34 % [6]. Other studies have demonstrated an overlap pattern in children with a mix of type 1 and type 2 histology. When Nobili and colleagues evaluated 84 children in Italy, an overlap pattern of these two histologic types was reported in 52 % of participants, whereas type 2 was reported in 27 %. In this group, ballooning was present in nearly half of the participants with the mixed histologic pattern [7]. A high prevalence of overlap in the two patterns of histology (82 %) was also found in a study of 130 pediatric participants with NAFLD. In this group, an equally high proportion of patients (85 %) had a pattern less common in children with zone 3 portal injury; 73 % had ballooning degeneration [8]. These differences may be due to racial and ethnic differences or differences in histologic interpretation, and more studies are needed. What is common between the studies, however, is that type 1 pattern is not predominant in pediatric patients as it is in adults.

Kleiner and colleagues developed the most commonly used NAFLD scoring system that is known as the NAFLD activity index or score (NAS), details of which are found in a previous chapter. The NASH Clinical Research Network Pathology Committee has validated this scoring system, and its intended use is for the assessment of change in clinical trials, not as a replacement for the diagnosis of steatohepatitis [9]. Of note, the NAS does not include portal inflammation, which is a common histologic feature in pediatric NAFLD. The NAS score does not correlate with histologic diagnosis [10] and thus should not be used for that purpose. In addition, this scoring system does not account for changes in histologic features over time.

Epidemiology

Prevalence

Determining the prevalence of NAFLD is hampered by the absence of accurate biomarkers. There have been a limited number of studies on NAFLD prevalence using the clinical reference standard of liver histology. The Study of Child and Adolescent Liver Epidemiology (SCALE) was a population-based autopsy study that included 742 children aged 2–19 years who had an autopsy for rapid out-of-hospital death between 1993 and 2003 in San Diego County. The age-, race-, gender-, and ethnicity-adjusted prevalence of fatty liver was found to be 9.6 % [1]. In a second autopsy-based study from Lower Silesia, Poland, steatosis was found in 5.3 % of 343 children aged 6 months to 18 years [11].

When liver histology is not available, studies have used alanine aminotransferase (ALT) as a surrogate marker for NAFLD. One study using data from the National Health and Nutrition Examination Survey (NHANES) evaluated more than 5500 adolescents between 1999 and 2004 and found that 8 % of participants had ALT > 30 U/L [12]. The Screening ALT for Elevation in Today's Youth (SAFETY) study used data from NHANES 1999–2006 to determine biologically based thresholds for the upper limit of normal (ULN) of ALT in healthy-weight, metabolically normal, liver disease-free children. These were determined to be 25 U/L in boys and 22 U/L in girls. Using these threshold values, for children aged 12–17, elevated ALT was present in 15.0 % of boys and 8.6 % of girls [13]. One additional study utilized NHANES to compare trends in ALT elevation over time. Using the threshold values from the SAFETY study, Welsh et al. reported that for children aged 12–19, the prevalence of an elevated ALT in children who were overweight or obese was 3.9 % in 1988–1994 and 10.7 % in 2007–2010 [14]. Pediatric NAFLD is a global problem. A study of 16,390 children aged 12.6 ± 2.6 years from Germany, Austria, and Switzerland reported a prevalence of ALT > 50 U/L of 12.4 % [15]. In the Korean

National Health and Nutrition Examination Survey from 1998, 3.2 % of children aged 10–19 had ALT>40 U/L [16].

Race and Ethnicity

Differences in the prevalence of NAFLD in various racial or ethnic groups have been noted. Hispanics have a higher prevalence of NAFLD than non-Hispanics. In SCALE, NAFLD was present in 11.8 % of Hispanic children, 10.2 % of Asian children, 8.6 % of White children, and 1.5 % of African American children [1]. For NHANES 1999–2004, the rates of ALT>30 U/L showed similar trends: 11.5 % of Hispanic children, 7.4 % of White children, and 6 % of African American children [12].

Gender

Boys have a higher prevalence of NAFLD than girls in almost all clinical and population-based studies of NAFLD [17–19]. In SCALE, the prevalence of NAFLD was 11.1 % in boys and 7.9 % in girls [1]. Similarly, data from NHANES years 1999–2004 reported that the rate of having ALT>30 U/L was 12.4 % in boys and 3.5 % in girls [12]. In contrast, the Western Australian Cohort (Raine) study is the only population-based study in which girls had a higher prevalence of suspected NAFLD than boys. In 1170 adolescents, the prevalence of increased liver echogenicity measured by ultrasound was 16.3 % in girls and 10.1 % in boys [20].

Age

In children the prevalence of NAFLD increases with age. The prevalence of NAFLD in SCALE was 0.7 % of children aged 2–4, 3.3 % of children aged 5–9, 11.3 % of children aged 10–14, and 17.3 % of children aged 15–19 [1]. Suspected NAFLD, as measured by ALT>35 U/L, was also shown to have an age-based increase in prevalence among 475 normal-weight and 517 overweight Hispanic children; elevated ALT was present in 15 % of 4–5-year-olds, 21 % of 6–11-year-olds, and 30 % of 12–19-year-olds [21].

Prevalence of NASH

In SCALE, NASH was present in 23 % of children with NAFLD [1]. In a separate study, NAFLD was present in 83 % of intraoperative biopsies from 41 morbidly obese adolescents undergoing bariatric surgery, among which 24 % had NASH [22]. A study by the NASH Clinical Research Network (NASH CRN) reported that 36 % of 176 children with biopsy-proven NAFLD had definite NASH [23]. In a recent study of 347 children ≥10 years old screened and referred to gastroenterology for suspected NAFLD by primary care providers, NASH was diagnosed in 30 % [18]. It is important to recognize that the prevalence of NASH may be greater in subspecialty-based samples because children with more severe disease are likely disproportionately represented than in population-based samples.

Pathogenesis

Most of what is known about the pathophysiology of NAFLD has been derived from animal models or adults. Moreover, the animal models were not pediatric specific. The content provided here is a brief overview of basic science and clinical studies limited to pediatrics.

Hedgehog Signaling

Hedgehog signaling regulates organogenesis and is silent in adult livers until injury induces hedgehog ligand production [24, 25]. Hedgehog pathway is necessary for adult livers to regenerate, but its activity also correlates with the severity of portal inflammation and fibrosis [26].

In children, the liver normally loses cells that produce or respond to hedgehog ligands. However, childhood NAFLD may interfere with

this process. Swiderska-Syn et al. evaluated 56 children with biopsy-proven NAFLD. Boys had higher portal/periportal hedgehog ligand production. Boys also had greater ductal proliferation ($p<0.05$) and more hedgehog-responsive portal cells ($p<0.017$) [27].

Insulin Resistance

Systemic insulin resistance, an impaired glucose response to insulin, is believed be important in the pathogenesis of pediatric NAFLD. In patients with NAFLD and insulin resistance, insulin does not suppress adipose tissue lipolysis as it does in healthy patients without insulin resistance [28]. This increased adipose lipolytic activity increases free fatty acids in circulation in patients with NAFLD. These circulating free fatty acids further impair insulin signaling by increasing insulin resistance in the skeletal muscle [29]. Circulating free fatty acids become the predominant source of intrahepatic triglycerides [30, 31]. To demonstrate impaired insulin-mediated suppression of adipose lipolysis, a study evaluated 18 obese adolescents who underwent magnetic resonance spectroscopy (MRS) for measurement of signal fat fraction, half of whom had signal fat fraction ≥10 %. Those who had insulin resistance and hepatic triglyceride content ≥10 % had increased adipose lipolytic activity and increased serum free fatty acid levels compared to participants with MRS signal fat fraction <10 % [32]. Additionally, children with insulin resistance have more de novo lipogenesis [33]. In summary, insulin resistance results in a combination of increased free fatty acid circulation and de novo lipogenesis, both of which have been implicated in the pathogenesis of NAFLD.

This increased prevalence of insulin resistance in children with NAFLD has been documented in several clinical studies. In a study by Schwimmer et al. describing the clinical characteristics of 43 children with biopsy-proven NAFLD, 75 % of study participants had fasting hyperinsulinemia, and 95 % of participants met criteria for insulin resistance based on the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) [34]. Moreover, in another study of 50 adolescents, half of whom were not obese, HOMA-IR > 2 was determined to be an independent risk factor for a hepatic signal fat fraction of >5.5 % as measured by MRI [35]. HOMA-IR levels were found to be significantly ($p<0.05$) higher among 41 obese Hispanic adolescents with MRI-determined hepatic signal fat fraction >5 % (8.8 ± 1.1) compared to those with MRI-determined hepatic signal fat fraction <5 % (5.5 ± 0.5) [36]. Thus, insulin resistance is associated with NAFLD. However, insulin resistance, in and of itself, is not sufficient for NAFLD development. In 4124 non-Hispanic African Americans aged 12–19, insulin resistance did not correlate well with ALT elevations >30 U/L (OR 3.93, $p<0.05$) [37].

In addition, African American children have been shown to have higher rates of obesity and cirrhosis, but do not have higher rates of elevated ALT. White children had a fourfold increase in ALT compared to African American children in a study of 181 obese children aged 4–17, 81 % of whom were non-Hispanic African American and 18 % were non-Hispanic white and 0.7 % were Hispanic white. The mean ALT values were significantly lower in black children (5.4 % of African Americans had ALT > 40) compared with white (21 % had ALT > 40) [38].

Inflammatory Cytokines

Resistin

Cytokines derived from adipocytes that promote systemic and hepatic insulin resistance and inflammation include TNF-α and resistin, which is a hepatic progenitor cell adipokine [39, 40]. These cytokines are involved in pathways that require activation of complex feedback loops. In a study of immunohistochemistry and immunofluorescence of 30 biopsies from pediatric patients with NAFLD (19 with NASH), resistin correlated with steatosis, inflammation, hepatocyte ballooning, and fibrosis. In children with NASH from the same study, resistin expression was correlated to the presence of fibrosis ($r=0.432$; $p<0.05$) [41]. However, in a study

with a larger sample size, Fitzpartick et al. demonstrated that resistin was significantly lower in children with NASH than isolated steatosis ($p=0.03$) in 40 children with biopsy-proven NAFLD [42]. More research is needed to better understand the expression of adipokines in the context of pediatric NAFLD pathophysiology as current studies have conflicting results.

PPAR-γ Signaling

Peroxisome proliferator-activated receptor-γ (PPAR-γ) signaling may have a protective effect for the liver against free fatty acids [43]. PPAR-γ is a type II nuclear receptor involved in mediating the ability of the adipocyte to adapt to overfeeding by hypertrophy. The role of PPAR-γ signaling was investigated in a population-based sample of 781 obese children in Taiwan with abnormal liver echogenicity measured by ultrasound. These children were genotyped to test the hypothesis that a single nucleotide polymorphism in the PPAR-γ co-activator (PGC)-1α gene (PPARCG1A) risk A allele (rs8192678) would influence the risk for hepatic steatosis. It was found that this allele was an independent risk factor for abnormally higher liver echogenicity determined by ultrasound with an odds ratio of 1.74. Subjects with this risk allele had a higher mean serum ALT (28.2 ± 31.1 U/L) compared to subjects without the allele (22.8 ± 22.2; $p=0.0006$) [44].

Leptin and Adiponectin

Activation of hepatic stellate and dendritic cells has been demonstrated to promote the progression of steatosis to steatohepatitis and hepatic fibrogenesis through signaling involving leptin. This cytokine can directly activate hepatic stellate cells by binding to their receptors or indirectly activate them through transforming growth factor-β secretion by Kupffer cells [45–47]. Leptin levels have been demonstrated to be elevated in obese children with abnormal liver echogenicity determined by ultrasound compared to obese children without increased liver ultrasound echogenicity [48].

Adiponectin, a protein secreted by adipocytes, is involved in fatty acid catabolism and glucose homeostasis [49]. With the aim of evaluating the relative concentrations of cytokines in children with elevated aminotransferases, Louthan compared 12 normal-weight children and 11 overweight children and found, after controlling for insulin and glucose, there was a moderate inverse correlation between adiponectin and serum ALT [50]. In a multiethnic cohort of 392 obese adolescents, those with signal hepatic fat fraction >5.5 % as measured by MRI had lower levels of adiponectin than obese adolescents with hepatic signal fat fraction <5.5 % [51].

Intestinal Microbiome

The intestinal microbiome may play a role in the pathogenesis of NAFLD. To explore this theory, a study of gut microbiome in 22 children with biopsy-proven NASH was compared to 25 obese children with normal liver function tests and 16 normal-weight children. There was an increased abundance of alcohol-producing bacteria (*E. coli*) noted in the microbiome of children with NASH and significantly elevated blood-ethanol concentrations in patients with NASH ($p<0.001$) compared to controls [52]. These findings, plus the known role of alcohol metabolism in oxidative stress and liver inflammation, suggest a role for alcohol-producing microbiota in the pathogenesis of NASH.

Genetics

Heritability

Factors that indicate a genetic component of NAFLD include the difference in prevalence between racial and ethnic groups and the clustering of NAFLD in families. To demonstrate the heritability of NAFLD, Schwimmer et al. evaluated 33 obese children with biopsy-proven NAFLD, 11 obese children without NAFLD, and 152 of their family members including parents, siblings, and second- or third-degree relatives [53]. MRI was used to assess the proton density hepatic fat fraction (PDFF) of family

members. In children with biopsy-proven NAFLD compared to those who did not have NAFLD, siblings of those with NAFLD were more likely to have MRI PDFF >5 % (59 % compared to 17 %). Parents of participants were more likely to have NAFLD than parents of those without NAFLD (78 % compared to 37 %). With 0 being no heritability and 1 indicating complete heritability, a heritability estimate of 0.85 was reported as the unadjusted dichotomous variable for NAFLD. After adjusting for age, gender, race, and BMI, the heritability estimate was 1.0. When considering hepatic steatosis as a continuous measurement, the unadjusted heritability estimate was 0.58, and the adjusted estimate was 0.39.

PNPLA3

Many genetic studies of NAFLD focus on the gene patatin-like phospholipase 3 (*PNPLA3*), which encodes the protein adiponutrin. A genome-wide association study (GWAS) resulted in the discovery of a common variant allele in *PNPLA3* (cytosine to guanine substitution), rs738409, that confers susceptibility to NAFLD [54]. Among other common variants identified through GWAS, the risk allele of *PNPLA3* stands out as the first polymorphism that has been very highly associated with the onset and progression of NAFLD independent of BMI, diabetes, or alcohol use. This variant protein is involved in lipid metabolism and may confer abnormal accumulation of triglycerides in the liver in patients carrying the risk allele. This section will focus only on studies of PNPLA3 in children with Table 17.1 provided as reference.

ALT and PNPLA3

Several studies have evaluated interactions between ALT and the variant allele in children. In a study of 475 obese or overweight children, 32 % of subjects with homozygous minor alleles for PNPLA3 (GG) had ALT values >30 U/L versus ALT>30 U/L in only 10 % of subjects who had the homozygous wild-type allele (CC) [55]. In a subsequent study of 520 obese Taiwanese children, higher ALT levels were found in children who were homozygous for the minor allele [56]. In a large Italian study, Giudice et al. evaluated 1048 obese children and found that there was a significant positive interaction between the variant allele and waist circumference with respect to risk for elevated serum ALT. Homozygotes showed a stronger correlation between ALT and waist to height ratio than heterozygotes [57]. In another large study evaluating 1037 Mexican children aged 6–12, the variant allele was found to be significantly associated with ALT levels >35 U/L. Once stratified by weight classification, there was a significant interaction between weight status and risk for ALT>35 U/L. In normal-weight children with the CC genotype, ALT elevation was extremely rare, but in normal-weight children with the GG genotype, the rate of ALT elevation was similar to obese children with the CC genotype [58].

Imaging, Histology, and PNPLA3

A study by Goran et al. evaluated 188 Hispanic children at the University of Southern California (USC) using MRI to evaluate hepatic signal fat fraction and found signal fat fraction in GG subjects was 1.7 and 2.4 times higher than GC and CC subjects (11.1±0.8 % in GG vs. 6.6±0.7 % in GC and 4.7±0.9 % in CC; $p<0.0001$) [25]. Santoro and colleagues reported similar findings in a study of 85 children from the Yale Pediatric Obesity Clinic. They reported a significant effect of the variant G allele such that those with at least one G allele had substantially greater hepatic signal fat fraction [59].

Conflicting evidence exists regarding the association between histologic severity and genotype in children. In 2010, Rotman and colleagues evaluated 223 children from the NASH CRN. In this study, there was no association of PNPLA3 with the histologic severity of NAFLD [26], contrary to what they saw in adults. In Italy, a study of 149 children with biopsy-proven NAFLD reported an association with the variant PNPLA3 allele and histologic severity. Valenti and colleagues reported that the variant G allele was associated with several key features of NAFLD including the severity of steatosis and

Table 17.1 Studies of PNPLA3 in children

Author	Comparators	*N*	Population	Findings
ALT				
Larrieta-Carrasco et al. [58]	ALT ≤ or >35 U/L	1037	Overweight Mexican children aged 6–12	PNPLA3 GG genotype had 3.7 times the odds for ALT>35 (95 % CI 2.3–5.9, $p=3.7\times10^{-8}$)
Giudice et al. [57]	ALT ≤ or >40 U/L	1048	Obese Italian children aged 2–16	PNPLA3 GG genotype had 2.97 times the odds for ALT>40 (95 % CI 1.80–4.18)
Romeo et al. [55]	ALT ≤ or >30 U/L	475	Overweight Italian children mean age 10 years ±3	ALT>30 U/L in 32 % with GG genotype and 10 % with CC genotype
Lin et al. [56]	Mean ALT	520	Obese Taiwanese children aged 6–18	Mean ALT was 31 U/L for GG genotype and 22 for CC genotype
Imaging via MRI				
Goran et al. [25]	MRI-determined signal fat fraction ≤ or >5.5 %	188	Hispanic American children aged 8–18	Mean signal fat fraction was 11 % for GG genotype and was 4.7 % for CC genotype
Santoro et al. [59]	MRI-determined signal fat fraction ≤ or >5.5 %	85	Clinically obese American children aged 8–18	Signal hepatic fat fraction >5.5 % overall; of those, 7 had CC vs. 32 with either CG or GG
Histologic severity				
Rotman et al. [26]	Histologic parameters	223	NASH CRN	PNPLA3 genotype was not associated with severity of steatosis, presence of NASH, or severity of fibrosis
Valenti et al. [60]	NASH and fibrosis	149	Italian children mean age 10.2±2.6	PNPLA3 G allele had 1.9 times the odds for fibrosis (95 % CI 1.14–3.45 per number of G alleles)
Diet				
Davis et al. [24]	MRI signal fat fraction ≤ or >5.5 %	153	Hispanic American children aged 8–18	With GG genotype, hepatic fat positively correlated with carbohydrate intake ($r=0.38$) and total sugar intake ($r=0.33$) but not with CG or CC genotypes
Santoro et al. [62]	Signal hepatic fat fraction ≤ or >5.5 %	127	Clinically obese American children mean age 14.7 years ±3.3	With GG genotype, hepatic fat positively correlated with n-6/n-3 PUFA intake ($r^2=0.45$) but not with CG or CC genotypes

the presence of steatohepatitis. In addition, the G allele was associated with the presence of pericentral fibrosis but not portal fibrosis [60]. Given the sample sizes of these studies and some conflicting observations, larger and more diverse studies will be needed to better evaluate the role of PNPLA3 on liver histology in children with NAFLD.

Diet and PNPLA3

Several groups have performed subsequent analysis of their data to evaluate the effect of diet on PNPLA3. The USC group evaluated the influence of PNPLA3 variants on hepatic fat modulated by carbohydrate and sugar intake in 153 Hispanic children. There was a mild but significant correlation between carbohydrate and/or sugar intake and hepatic signal fat fraction only in those children who were homozygous for the G allele [24]. However, when histologic variables were assessed in a study of 149 children with biopsy-proven NAFLD, sugar-sweetened beverage consumption was not associated with the histologic features nor the severity of NAFLD [61]. The Yale group evaluated 127 pediatric participants with a median age of

14.7 ± 3.3 whose dietary composition was assessed for essential omega polyunsaturated fatty acid (PUFA) intake. In this study there was a moderate but significant correlation between dietary PUFA intake and hepatic signal fat fraction only in those children who were homozygous for the G allele [62].

Influence of Maternal Factors and Breastfeeding on NAFLD

The possibility that NAFLD may start at birth has been explored by looking at infants of obese and diabetic mothers. In a study of 25 neonates born to normal-weight mothers ($n = 13$) and obese mothers with gestational diabetes ($n = 12$), Brumbaugh and colleagues evaluated differences in neonatal fat distribution. Neonates underwent MRI measurement of subcutaneous and intra-abdominal fat and magnetic resonance spectroscopy (MRS) for signal hepatic fat fraction at 1–3 weeks of age. Infants born to obese mothers with gestational diabetes had a mean 68 % greater liver hepatic signal fat fraction compared to infants born to normal-weight mothers. In all infants, signal hepatic fat fraction correlated with maternal prepregnancy BMI but not with subcutaneous adiposity [63].

Modi and colleagues studied 105 mother/neonate pairs to determine whether neonatal liver signal fat fraction measured by MRS was influenced by maternal BMI. They found a strong relationship between prepregnancy BMI and infant signal fat fraction even after adjusting for infant sex and postnatal age; women with higher BMI had babies with higher signal fat fraction [64].

Once a child is born, neonatal overfeeding may have a long-term effect on de novo lipogenesis [65]. Breastfeeding may be protective. In an investigation of 191 Caucasian children with biopsy-proven NAFLD, the distribution of steatosis, inflammation, hepatocyte ballooning, and fibrosis were all worse among children who were not breastfed compared to breastfed children [66].

Clinical Features

Obesity

There is a strong association between obesity and NAFLD in the pediatric population. As many as 70–90 % of children with NAFLD are obese [67]. In SCALE, the prevalence of NAFLD was 5 % among normal-weight children, 16 % among overweight children, and 38 % among obese children [1]. The distribution of adiposity is also important. Manco and colleagues reported that in 197 children with biopsy-proven NAFLD, 84 % had a large waist circumference (>90th percentile for age and gender). The only risk factor for liver fibrosis in this study was a large waist, and thus, body fat distribution may also be an important prognostic factor for disease severity [68].

The relationship between NAFLD, NASH, and morbid obesity in adolescents is less clear. In the Teen LABS study of 242 severely obese adolescents (mean BMI 50.5 kg/m^2) undergoing weight loss surgery, NAFLD was diagnosed or suspected in 37 % [69]. In a separate smaller study, Holterman and colleagues compared morbidly obese adolescents to morbidly obese adults. Among 24 severely obese adolescents undergoing weight loss surgery compared to 24 adults with similar BMI, severely obese adolescents had a significantly higher prevalence of NASH (62.5 % versus 25 %) and fibrosis (83 % versus 29 %) [69, 70].

It is important to highlight that NAFLD does occur and has been reported in children of normal weight, with a prevalence of 5–20 % [5, 71–73]. Not all children with NAFLD are obese, and not all obese children have NAFLD.

Acanthosis Nigricans

Acanthosis nigricans, a marker of hyperinsulinemia, is common in children with NAFLD. In acanthosis nigricans, the skin at the nape of the neck and sometimes in the axillary area appears hyperpigmented and thickened. Acanthosis nigricans has been found in 36–49 % of children with biopsy-proven NAFLD [34, 74].

Other Physical Exam Findings

Many patients with NAFLD have hepatomegaly. Thus, percussing and palpating the liver and estimating its size are important parts of the pediatric abdominal exam. The excess abdominal girth makes this difficult in obese children, but can still be performed effectively. There may be right upper quadrant tenderness as well as stigmata of chronic liver disease, such as scleral icterus and jaundice, gynecomastia, spider angiomata, palmar erythema, and asterixis.

Diagnostic Approaches

Screening

Screening children for NAFLD has been addressed by multiple societies (Table 17.2). In 2007, an expert committee on childhood obesity published guidelines on the assessment of overweight and obese children. They recommended screening for NAFLD in children who are ≥10 years old and obese or overweight with additional risk factors by measuring ALT and AST levels. The recommendations suggest consultation with a pediatric gastroenterologist should these results be ≥2 times the upper limit of normal (ULN) [75].

Since screening relies in part on lab values, understanding the "normative" range for these values is important. The median value for ALT upper limit of normal in children's hospitals nationwide is 53 U/L [13]. However, the SAFETY study identified that among healthy-weight children who did not have liver disease, a more biologically based 95th percentile for ALT was 25.8 U/L in boys and 22.1 U/L in girls. Thus, a lab value of 53 U/L that is considered within the normal reference range at many hospitals would be two times the ULN of the biology-based value. Since many labs continue to use inappropriate normal reference ranges, calculating two times the upper limit of "normal" as screening threshold to detect liver disease is problematic. Moreover, the optimal value of ALT that has sufficient accuracy to be used explicitly for NAFLD screening has not been agreed upon.

In a study of 347 overweight and obese children ≥10 years of age referred from primary care to pediatric gastroenterology for suspected NAFLD identified by screening, nearly 85 % of the children with a screening ALT ≥ 80 U/L (a value most often considered to be 2× ULN) had some form of liver disease. Although a majority of all of the children screened had NAFLD (53 %; 193/347), a nontrivial minority (18 %) of those referred had other forms of liver disease. In addition, 11 % of children referred were found to

Table 17.2 Society guidelines regarding screening overweight and obese children for NAFLD

	Recommend screening children for NAFLD			
Society	Yes	No	Uncertain	Not stated
American Academy of Family Physicians	X			
American Academy of Pediatrics	X			
American Association for the Study of Liver Disease			X	
American College of Gastroenterology			X	
American Gastroenterological Association			X	
Endocrine Society	X			
European Society for Pediatric Gastroenterology, Hepatology, and Nutrition	X			
National Association of Pediatric Nurse Practitioners	X			
North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition				X

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have advanced fibrosis at diagnosis [18]. Thus, implementing the recommended screening strategy identifies many children with previously undetected liver disease. However, it casts a wide net, as elevated ALT is not specific for the diagnosis of NAFLD. Moreover, ALT of ≥80 U/L was found to have a sensitivity of 57 % and a specificity of 71 % for diagnosing NAFLD. In contrast, when two times the biologically based thresholds of ALT were used, ALT ≥ 50 in boys and ≥44 in girls, there was a substantial increase in the sensitivity of diagnosis of NAFLD to 88 %; however, the specificity declined to 26 % [18].

This recent data does support the practice of screening with ALT to identify liver disease, although it illustrates that not every obese child with elevated ALT will have NAFLD. In addition, the optimal ALT cutoff for screening with sufficient accuracy to detect NAFLD in all children, including those with advanced fibrosis, is not clear. Careful history, physical exam, lab evaluation, and histologic evaluation are extremely important for making a correct diagnosis. Consideration of other causes of chronic liver disease in children, such as autoimmune hepatitis, drug toxicity, infectious hepatitis, Wilson disease, alpha-1-antitrypsin deficiency, celiac disease, hemochromatosis, and metabolic disease, must be considered in clinically appropriate situations.

Biomarkers

A noninvasive, sensitive, and specific biomarker for NAFLD would be helpful as many children with NAFLD go undiagnosed in part due to the requirement for a diagnostic liver biopsy. The most helpful biomarker would not only accurately reflect the presence of disease, but also classify severity of the disease along with the stage of fibrosis. Many biomarkers have been studied in children thus far. As shown in Table 17.3, there are several molecules that are associated with various histologic features of NAFLD; however, none of these have a sufficiently strong enough relationship to be considered clinically useful. For example, in the study of cytokeratin-18 (CK-18), information gathered in cross section or longitudinally does not appear to provide additional information different from what can be gained from measuring ALT [76]. Hyaluronic acid may be a better marker than human cartilage glycoprotein-39 (YKL-40) [77] and has a significant relationship to fibrosis [78], but it is unclear whether these findings may be clinically useful. Adipokines such as chemoattractant protein-1 (MCP-1) and plasminogen activator inhibitor-1 (PAI-1) have a significant relationship to fibrosis [42], yet it may not be strong enough for clinical utility. And finally, fibroblast growth factor-21 (FGF-21) had a mild positive correlation with liver fat ($r^2 = 0.278$) [79]. Although there is a significant association with biomarkers and histologic features in group aggregate, the findings are not sensitive nor specific enough to be used clinically.

Liver Imaging

There are a limited number of studies in children comparing radiologic techniques to the reference standard of histology in children with NAFLD. Currently, there are no radiologic techniques that have been shown to be diagnostic, though some show promise for screening and monitoring. The radiologic evaluation of hepatic fibrosis has also been attempted in limited studies involving children, but the abnormalities identified are of very advanced disease. Thus, their evaluation does not contribute to early detection, which is an important consideration in pediatrics.

Ultrasound Compared to Liver Histology

Ultrasound technology uses characteristics of high-frequency sound wave propagation in attempts to differentiate fatty tissue from normal hepatic tissue. Fatty tissue scatters the ultrasound beam, causing more echoes to return to the ultrasound transducer and resulting in a brighter more echogenic liver.

Table 17.3 Potential biomarkers in pediatric NAFLD

Author	Marker(s)	Sample size	Population	Comparator	Key findings
Lee et al. [77]	Hyaluronic acid (HA) Human cartilage glycoprotein-39 (YKL-40)	128	128 children and young adults aged 1.4 months to 27.6 years	Histology	**HA**: median 74.7 ng/mL in F3–F4 vs. 17.7 ng/mL in F0–F2 fibrosis ($p<0.0001$) **YKL-40**: median 31.5 ng/mL in F3–F4 fibrosis vs. 34.2 ng/mL in F0–F2 fibrosis ($p=0.85$)
Giannini et al. [79]	Fibroblast growth factor-21 (FGF-21)	217	Lean and obese adolescents	Fast gradient MRI to measure hepatic signal fat fraction (SFF)	FGF-21 levels correlated with HF $r^2=0.278$, $p<0.001$
Fitzpatrick et al. [42]	Monocyte chemoattractant protein-1 (MCP-1) Plasminogen activator inhibitor-1 (PAI-1)	40	Children recruited from a tertiary care pediatric hepatology unit	Histology	Predictors of advanced fibrosis : **MCP-1**: AUC 0.76 (95 % CI 0.62, 0.91) **PAI-1**: AUC 0.78 (95 % CI 0.6, 0.91)
Vuppalanchi et al. [76]	Cytokeratin-18	152	Children with NAFLD in TONIC	Histology	Change in histology: AUC 0.72 (95 % CI 0.63–0.81)
Lebensztejn et al. [78]	Cytokeritin-18 M30 Hyaluronic acid	52	Children	Histology	**CK-18 M30**: 177.5 U/L without fibrosis, 311 U/L with fibrosis ($p=0.05$) **HA**: 18.5 ng/mL without fibrosis, 20.5 ng/mL ($p=0.04$) **HA** (cutoff 19.1): sensitivity 84 %, specificity 55 %, PPV 52 %, NPV 86 %. AUC with fib = 0.672, AUC without fib = 0.666 **HA + CK-18**: sensitivity 74 %, specificity 79 %, PPV 56 %, NPV 63 %, AUC 0.73)

Although ultrasound is commonly used in routine practice to determine the presence and degree of fatty liver, a systematic review revealed its limitations in both of these areas [80]. The positive predictive value of liver ultrasound for the detection of fatty liver in children was found to be between 47 and 62 % [81, 82]. Thus, it is not an optimal modality to be used as a diagnostic test. This limitation is due to an inherent property of ultrasound, in that it does not measure fat directly, but relies on a subjective and nonquantitative interpretation of the echogenicity. Therefore, relying on ultrasound as a semiquantitative measure of hepatic steatosis is unreliable. In addition, the common practice of using ultrasound to exclude fatty liver has insufficient evidence. There has been only one study evaluating children who had a negative ultrasound in addition to a liver biopsy for comparison [82]. In that study, most of the negative ultrasounds turned out to be falsely negative. This was an artifact of the study design, as the study only included children

with known NAFLD. Thus, data are also lacking regarding the ability of ultrasound to exclude fatty liver in children. The primary role of ultrasound in pediatrics is in the evaluation of structural problems within the liver or gallbladder. Future studies using ultrasound should consider the evaluation of emerging quantitative ultrasound techniques using liver biopsy as the reference standard for diagnosis and grading hepatic steatosis in children.

MRI Compared to Liver Histology

In pediatric clinical research studies, MRI has overtaken ultrasound as the modality of choice for the noninvasive measurement of hepatic steatosis. In some institutions, MRI is now used in standard clinical practice to measure hepatic signal or proton density fat fraction. The increasing use of MRI is due to its growing availability and promise as a quantitative measure of hepatic steatosis. However, the evidence base is extremely limited. One study evaluated 25 obese children in Rome, Italy, with biopsy-proven NAFLD who underwent MRI prior to liver biopsy to explore the accuracy of the MRI-determined hepatic signal fat fraction [83]. The MRI method used in this study was a modification of the 2-point Dixon method [84]. The MRI-determined hepatic signal fat fraction was strongly correlated ($r=0.88$) with the histological grade of steatosis (grade 0 <5 %, grade 1 5–33 %, grade 2 34–65 %, grade 3 ≥66 %). The small sample size precluded more specific determinations of accuracy.

An advancement in the field beyond common use of the modified 2-point Dixon method has identified the importance of performing MRI correctly to compensate for confounders that introduce error into both the accuracy and precision of conventional MRI [85–90]. Such methods are referred to here as advanced MRI. A recent study of advanced MRI compared to histology in 174 children demonstrated that an advanced MRI measure of steatosis called proton density fat fraction (PDFF) correlated well with histologically determined steatosis grade in children. MRI-estimated liver PDFF was significantly ($p<0.01$) correlated (0.725) with steatosis grade. The correlation was significantly ($p<0.01$) stronger in girls (0.86) than in boys (0.70) and was significantly ($p<0.01$) weaker in children with stage 2–4 fibrosis (0.61) than children with no fibrosis (0.76) or stage 1 fibrosis (0.78). This study also evaluated published magnetic resonance-derived threshold values intended to discriminate between no steatosis and mild steatosis (1.8 %, 5.5. %, 6.4 %, and 9 %). Sensitivity ranged from 42 to 98 %, while specificity ranged from 54 to 96 % [91]. Achieving a distinct separation between having and not having a fatty liver based upon a single MRI-based cutoff point remains challenging.

Treatment

Lifestyle Interventions

Pediatric NAFLD is often associated with obesity; thus, dietary and exercise treatments are often recommended. NAFLD intervention trials have focused on weight loss, but data for lifestyle modification specifically in children with NAFLD are limited [7, 92–98]. Moreover, the specific amount of weight loss required that will result in an improvement in NAFLD in children is not clear. Histology is the cornerstone of diagnosis and should be used as an outcome measure to assess the efficacy of interventions, but current outcome measures in lifestyle treatment studies vary. One of the early studies by Nobili and colleagues enrolled 84 obese or overweight children with biopsy-proven NAFLD to evaluate the effect of low-calorie diet and individually tailored moderate exercise. A total of 52 participants completed the trial, which included medical examinations and laboratory assessment at 3-month intervals and an ultrasound at 12 months. Seventeen participants lost >10 % body weight. Only 5 of the 17 children with >10 % weight loss had a normal ultrasound at 1 year [7]. However, histologic change was not assessed. The greatest decrease in ALT was seen in those who lost 5 % or more of their body weight. At this time, weight loss with diet and exercise should continue to be

recommended for overweight and obese children. However, weight loss alone may not be sufficient to improve NAFLD in all children, and further research with histology as an outcome measure is certainly needed.

There is interest in the role of fructose in the pathogenesis of NAFLD. Fructose consumption has been suggested to be a risk factor for NAFLD [99, 100]. A pilot study evaluated the efficacy of a low-fructose diet in decreasing ALT in children with NAFLD. Ten children with NAFLD or suspected NAFLD were placed on either a low-fructose diet ($n=6$) or low-fat diet ($n=4$) for 6 months. There was no significant change in ALT in either group. Histologic changes in NAFLD were not assessed [94]. Subsequently, Vos et al. conducted a 4-week, double-blind, randomized, controlled intervention study of 21 Hispanic children aged 11–18 with BMI $\geq$ 85 % who regularly consumed sweet beverages. All participants had hepatic fat quantification using MRS signal fat fraction. They were randomized to drink 24 fluid ounces of fructose or glucose beverage per day. After 4 weeks, there was no significant change in hepatic fat in either group [101]. In summary, while fructose can cause NAFLD in animal models, the degree to which fructose is a relevant factor in children with NAFLD remains uncertain.

Pharmacologic Therapy

Antioxidant and Hepato-protective Agents

Mitochondrial dysfunction and damage by reactive oxygen species are implicated in the pathogenesis of NAFLD as described in previous chapters, and thus, antioxidants have been evaluated as a potential therapy. Treatment with vitamin E resulted in decreased ALT in a pilot study of 11 children with increased liver echogenicity determined by ultrasound [102]. Vitamin E as an intervention was again studied in a larger cohort of 88 children with biopsy-proven NAFLD who also participated in monthly sessions with dieticians. In this study, Nobili and colleagues compared children receiving vitamin E versus vitamin C versus placebo for 1 year. Treatment with vitamin E did not significantly improve ALT compared to vitamin C or placebo [103]. In a follow-up study by the same group, about 60 % of these patients underwent liver biopsy. Although there was significant improvement in steatosis, hepatocellular ballooning, and lobular inflammation among study participants, there was no significant difference in histology between vitamin E and placebo groups [104]. Subsequently, the Treatment of NAFLD in Children (TONIC) trial, a large NASH CRN multicenter, randomized, double-blind, placebo-controlled trial, was completed in 2010 [105]. In this trial, 173 children with biopsy-proven NAFLD were randomized to receive metformin, high-dose vitamin E, or placebo for 96 weeks. The primary outcome measure was a decrease in ALT by 50 % compared to baseline or a decrease to less than 40 U/L. In that study, treatment with vitamin E did not result in significant decrease in ALT compared to placebo. In addition, the features of steatosis inflammation, ballooning, and fibrosis were evaluated after 2 years of treatment. There was no significant improvement in steatosis, inflammation, or fibrosis with vitamin E. However, hepatocyte ballooning was shown to improve in 38 % (22/58) of children taking vitamin E and only 17 % (10/58) of children taking placebo.

Ursodeoxycholic acid (UDCA), a cytoprotective agent, is a secondary bile acid formed by intestinal bacteria. In one study of 31 obese children with elevated ALT and increased echogenicity as determined by liver ultrasound, the effect of UDCA on ALT was determined. Participants were divided into four groups: diet alone ($n=11$), UDCA treatment ($n=7$), UCDA and diet ($n=7$), and untreated controls ($n=6$) [106]. UDCA alone was not effective in lowering ALT.

Cysteamine is an aminothiol agent that acts as an antioxidant by scavenging reactive oxygen intermediates as well as increases glutathione, the most abundant intracellular antioxidant agent, and thus is a candidate therapy for NAFLD [107]. In an open-label pilot study of 11 children with biopsy-proven NAFLD and serum ALT $\geq$ 60 U/L, Dohil et.al evaluated the effect of twice-daily enteric-coated cysteamine for 24 weeks on serum

ALT [107]. At the 24-week time point, 64 % of subjects had a decrease in serum ALT by at least 50 % of baseline. This effect was maintained at 48 weeks. This cohort was evaluated to assess the impact of cysteamine treatment on multimerization. After 24 weeks of therapy, there was an increase in total adiponectin (49.3 %, $p=0.05$) from baseline [108]. Currently, the NASH CRN is conducting a multicenter, placebo-controlled clinical trial of children aged 8–17 years with biopsy-confirmed moderate to severe NAFLD. The primary objective is to evaluate whether 52 weeks of treatment with cysteamine bitartrate delayed-release capsules will result in an improvement in liver disease severity [109].

Targeting Insulin Resistance with Metformin

Insulin resistance is believed to be a key component in the pathogenesis of NAFLD. Therefore, the efficacy of metformin has been studied as a treatment option for pediatric NAFLD. In an open-label pilot study of ten children with biopsy-proven NASH, metformin treatment was evaluated using MRS signal fat fraction [110]. Normalization of ALT occurred in 40 % of subjects. There was also a significant reduction in hepatic signal fat fraction in 90 % of subjects from a mean of 30–23 % after 24 weeks of treatment. Nobili and colleagues also conducted an open-label pilot study. In this study, 30 children with biopsy-proven NAFLD underwent 24 months of metformin treatment [111]. Of the 40 % of participants who had a follow-up biopsy, several histologic features including steatosis, ballooning, and lobular inflammation improved after metformin treatment. In the TONIC trial, oral metformin treatment of 500 mg twice daily for 96 weeks did not result in a significant decrease in ALT compared to placebo. In addition, the features of steatosis inflammation, ballooning, and fibrosis were evaluated after 2 years of treatment. There was no significant improvement in steatosis, inflammation, or fibrosis with metformin. However, hepatocyte ballooning was shown to improve in 39 % (22/57) of children taking metformin and only 17 % (10/58) of children taking placebo [105].

Dietary Supplements

Limited studies are available assessing the efficacy of dietary supplements, such as omega-3 polyunsaturated fatty acids and probiotics, for treatment of NAFLD in children. One randomized clinical trial evaluating treatment with docosahexaenoic acid (DHA) enrolled 60 children with biopsy-proven NAFLD for 6 months. Subjects were randomized to one of three groups: DHA 250 mg/day, DHA 500 mg/day, or placebo. Treatment with DHA did not improve serum ALT or BMI, but was noted to improve insulin sensitivity [112].

Whether manipulation of the microbiome can impact pediatric NAFLD status is unclear, as there are only a few double-blind, placebo-controlled studies of probiotics in children with NAFLD that have been carried out to date. One such study enrolled 20 obese children with abnormal hepatic echotexture on ultrasound and elevated transaminases that persisted for >3 months. They were randomized to treatment with *Lactobacillus* GG (12 billion CU/day) for 8 weeks or placebo. ALT was noted to decrease significantly from 70 U/L ± 35 to 40 U/L ± 22 in the lactobacillus group over the 8-week study period, whereas in the control group, ALT remained unchanged [113]. The authors noted there was no change in echogenicity measured by ultrasound in either group. In another randomized double-blind placebo-controlled clinical trial, 22 Caucasian children with biopsy-confirmed NAFLD whose median age was 10 years were treated with a proprietary blend of probiotics and compared to 22 children given placebo. There was no significant change in ALT or insulin sensitivity noted in either group. Interestingly, a decrease in BMI of 8 % was noted in the treatment group, while there was no change in BMI in the placebo group [114]. Whether any liver-related feature of NAFLD improves with probiotics is yet to be determined.

Surgery

Bariatric surgery has become an important treatment option for morbidly obese patients and is increasingly used in the adolescent population.

Since NAFLD is associated with obesity, bariatric surgery may provide a mechanism for treating NAFLD. Studies in the pediatric population are available that report on significant weight loss and decreased liver chemistries in adolescents who underwent laparoscopic adjustable gastric banding [115, 116], but they do not report data on histologic resolution of NAFLD after bariatric surgery. More studies are needed to determine the efficacy and safety of bariatric surgery to treat pediatric NAFLD.

Outcomes

Children with NAFLD have many associated comorbidities. Outcome data, however, are lacking due to the small number of longitudinal pediatric studies performed to date.

Mortality

Outcome data for pediatric NAFLD are limited with respect to mortality. One study following 66 children with NAFLD for a mean follow-up time of 6.4 years found children with NAFLD to be at higher risk for mortality compared to the general population with a standardized mortality ratio of 13.6 %; two of these children required liver transplant for decompensated cirrhosis, and two died from non-liver-related conditions [117].

Advanced Fibrosis and Cirrhosis

Advanced fibrosis has been reported in 5–15 % of children with biopsy-proven NAFLD at the time of diagnosis [23, 60]. Fibrosis can progress rapidly in some children. For example, in 102 children with NAFLD, the prevalence of advanced fibrosis increased to 20 % after a median follow-up of 2.2 years [118]. Cirrhosis and its sequelae have been observed in children with NAFLD [119].

Hepatocellular Carcinoma

When NAFLD begins in childhood, the long duration of disease raises concern for the future risk of hepatocellular carcinoma. HCC in association with NAFLD has been reported as young as age 7 [41]. Screening efforts should rightfully be directed at those children with NAFLD who have advanced fibrosis. Important clinical issues to address include deciding which children to screen for HCC and which method to use for screening and determining the optimal frequency of screening.

Cardiac Complications

Children with NAFLD have an increased risk for development of cardiovascular disease that has been demonstrated through the evaluation of dyslipidemia, hypertension, arterial stiffness, left ventricular mass, and direct measures of cardiac function.

Dyslipidemia

Dyslipidemia is common in children with NAFLD. Among 120 children with biopsy-proven NAFLD from Italy, 46 % had HDL cholesterol < 5th percentile for age and sex, and 63 % had triglycerides >95th percentile for age, gender, and race [72]. In 150 children with biopsy-proven NAFLD from California, 46 % had triglycerides >150 mg/dL compared to only 12 % of obese children without NAFLD matched for age, sex, and BMI [120]. Not only are rates of dyslipidemia high in children with NAFLD, but hepatic steatosis in children is specifically associated with a more atherogenic lipid profile. For example, among 49 obese adolescents divided into groups based on an MRS signal hepatic fat fraction cutoff point of 5.5 %, those with signal hepatic fat fraction >5.5 % had significantly higher concentrations of small dense low-density lipoprotein particles ($p < 0.0007$) [121].

Hypertension

Elevated blood pressure is common in children with NAFLD independent of obesity [120, 122]. In a study of 150 overweight children with biopsy-proven NAFLD, children with NAFLD had significantly higher systolic and diastolic blood pressure than children without NAFLD [120]. In a recent study of 494 children in the

NASH CRN, the estimated prevalence of high blood pressure at baseline was 36 %, and persistent high blood pressure (at baseline and 48 weeks) was 21 %. Children with high blood pressure had significantly more severe steatosis (mild 19.8 %, moderate 35 %, severe 45.2 %) than children without high blood pressure (mild 34.2 %, moderate 30.7 %, severe 35.1 %) [123].

Left Ventricular Mass

One pediatric study evaluated 80 obese adolescents admitted to a pediatric endocrinology unit compared to 37 lean controls. Among the obese participants, 44 had elevated ALT and abnormal hepatic echogenicity measured by ultrasound compared to 37 lean controls. Obese adolescents with elevated ALT and abnormal echogenicity as determined by ultrasound had significantly higher left ventricular mass and lower insulin sensitivity as measured by HOMA-IR compared to both obese adolescents with ALT < 40 and normal liver echotexture and compared to lean controls [124].

Cardiac Function and Pediatric NAFLD

Studies have evaluated the association between pediatric NAFLD and cardiac dysfunction. In one study, 44 obese adolescents with hepatic signal fat fraction ≥5.6 % measured by MRS were compared to obese individuals with hepatic signal fat fraction <5.6 % in addition to lean controls. Left ventricular global longitudinal strain was significantly decreased in obese subjects with hepatic signal fat fraction >5.6 % compared to obese subjects with hepatic signal fat fraction <5.6 % and lean subjects, indicating greater systolic dysfunction [125]. Early diastolic longitudinal strain rates were also significantly decreased in obese subjects with hepatic signal fat fraction >5.6 % indicating greater diastolic dysfunction. Hepatic signal fat fraction measured by MRS was positively correlated with indicators of systolic and diastolic dysfunction (r : 0.25–0.40) [126].

Pacifico and colleagues evaluated 108 obese children to determine whether MRI hepatic signal fat fraction was associated with subclinical left ventricular structural and functional abnormalities independent of metabolic risk factors. Participants were divided into two groups: 54 who had MRI-determined signal fat fraction ≥5 % and 54 who had hepatic signal fat fraction ≤5 %. These groups were compared to 18 lean subjects. Forty-one of these children had biopsy-proven NAFLD, and 63 % (26/41) had definite NASH. Those with definite NASH had significantly lower e′ velocity and significantly higher E-to-e′ ratio (indicators of ventricular filling pressures) and lower Tei index (Doppler-derived index of combined systolic and diastolic myocardial performance) than those without NASH [127].

Obstructive Sleep Apnea

Symptomatic obstructive sleep apnea (OSA) includes daytime sleepiness, poor school performance, and snoring. Patients who become hypoxic during OSA are at risk for increased oxidative stress that may trigger a progression from steatosis to NASH. This is thought to be related to a mechanism involving ischemia and reperfusion tissue injury [128, 129]. Sundaram and colleagues explored evidence of this mechanism in children. In 25 obese children with biopsy-proven NAFLD who underwent polysomnography, OSA was present in 60 % of all subjects, and subjects with OSA had significantly more hepatic fibrosis than those without OSA [130]. In a larger study, Nobili and colleagues evaluated 65 children with biopsy-proven NAFLD to explore the relationship between OSA and biochemical, immunohistochemical, and histologic features of NASH and fibrosis. In this group, the prevalence of OSA was also 60 %. OSA prevalence and severity were associated with the presence of NASH (OR 4.89), fibrosis stage F ≥ 2 (OR 5.91) [131]. Interestingly, this relationship held true even among nonobese children with NAFLD. OSA is an important comorbidity to consider in children with NAFLD.

Endocrine

NAFLD is associated with conditions related to endocrine dysfunction such as diabetes,

alterations in bone mineral density, and hypovitaminosis D.

The most likely factor responsible for the associations between diabetes and NAFLD is insulin resistance, which is both part of the pathogenesis of NAFLD and plays a role in the development of type 2 diabetes. In a retrospective chart review of 115 children with type 2 diabetes, when ALT was used as a surrogate marker for NAFLD, 50 % of children with type 2 diabetes had suspected NAFLD [132]. In a study of 571 obese children, 41 % had increased echogenicity determined by ultrasound; 25 % of those with increased echogenicity had either impaired glucose tolerance or diabetes [133]. Other studies have noted that, at the time of diagnosis of biopsy-proven NAFLD, 8–10 % of children had type 2 diabetes [34, 134].

Pediatric NAFLD is also associated with alterations in bone mineral density (BMD). In a study of 38 children with biopsy-proven NAFLD, BMD as measured by DEXA was significantly lower in obese children with NAFLD compared to obese controls without NAFLD. It was also significantly lower in children with NASH compared to children with NAFLD but not NASH [135]. Among 44 obese children with an MRI signal fat fraction ≥5 %, BMD *z*-scores of the lumbar spine were significantly lower (mean 0.55) compared to 44 children whose MRI signal fat fraction was <5 % (mean 1.29) [136]. In a subset of children who had a liver biopsy, those with NASH had significantly lower BMD than those without NASH [136]. Thus, obese children with NAFLD may be at greater risk for fractures.

Children with NAFLD are at increased risk for low vitamin D 25-OH levels. This was demonstrated by Manco et al. who evaluated 64 children with biopsy-proven NAFLD in whom the mean level of 25(OH)D3 was 21.9 ± 10.2, and half of the study population (35/64) had levels of 25(OH) D3 below 20 ng/mL [137]. Conflicting data exists regarding the relationship between vitamin D levels and severity of liver disease. In a study of 73 overweight and obese children with biopsy-proven NAFLD, vitamin D 25-OH levels were found to be 9 ng/mL lower in children with NASH compared to those without NASH ($p<0.001$) [138]. In a study by the NASH CRN, however, 102 children with biopsy-proven NAFLD were studied, and there were no significant associations between the vitamin D level and the histologic features or severity of NAFLD [139].

Psychological

Children with NAFLD may also have substantial psychological burden. In a NASH CRN study that evaluated 239 children from eight clinical centers with biopsy-proven NAFLD, 39 % of children reported an impaired quality of life. In this study, nearly half of the variance in quality of life scores in participants with NAFLD compared to normal controls was accounted for by reports of fatigue, trouble in sleeping, and sadness [17]. In a case control study of depression in 48 obese children with NAFLD or suspected NAFLD compared to obese controls, children with NAFLD or suspected NAFLD had higher levels of depression on the Children's Depression Inventory compared to the 40 obese controls [140]. Therefore, screening for symptoms of depression and other indicators of low quality of life should be considered in the assessment of children with NAFLD.

Summary

Pediatric NAFLD is a global problem. It is a complex disease with pathogenic features that make it unique from adult NAFLD. At this time the exact interplay between insulin resistance, inflammatory cytokines, free fatty acids, adipokines, and genetic variation is yet to be understood, but there is evidence that maternal and neonatal factors may influence the development of NAFLD in children. Clinical and laboratory features of NAFLD in children are nonspecific; thus, diagnosis by histology remains the clinical standard. Many biomarkers have been studied in pediatric NAFLD, but data are limited and not strong enough to support the use of these biomarkers clinically. Among the available

noninvasive imaging modalities for assessing for the presence of NAFLD and the grading of its severity, the use of ultrasound is not supported, while the use of MRI shows promise. Current treatment options are focused on lifestyle interventions, while clinical treatment trials are underway. Effective treatment is important, as children with NAFLD are at risk for multi-organ complications. Greater understanding of the disease and its associated morbidities will help reduce the overall disease burden in children who are currently at risk for reduced life expectancy. Large, randomized, controlled trials in children using biopsy as a reference standard are needed in all areas. Fortunately, this innovative research is currently in progress to improve understanding and management of NAFLD in children.

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Index

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