Non-Alcoholic Fatty Liver Disease

A 360-degree Overview

Elisabetta Bugianesi
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To our cherished colleague and friend Valerio Nobili, a brilliant scientist and an extraordinary person.

Preface

The term "nonalcoholic fatty liver disease" (NAFLD) was coined by gastroenterologists almost 20 years ago to define a spectrum of progressive liver disease that encompasses simple steatosis, nonalcoholic steatohepatitis (NASH, which is characterized by the presence of steatosis, necroinflammation, with/without fibrosis), and ultimately cirrhosis. The same entity was also well known to diabetologists and regarded as an epiphenomenon of the metabolic syndrome. Dramatic changes in the lifestyle of the global population have been fueling a worldwide increase of obesity and its comorbidities, including NAFLD. It is estimated that the burden of end-stage liver disease will increase two- to threefold in both Western nations as well as several Asian countries by 2030, and NAFLD is set to replace viral hepatitis as the primary cause of end-stage liver disease and liver transplantation over the next decade or so, with the disease affecting both adults and children. It is clear that NAFLD is a complex disease, with considerable variation in severity among individuals as a result of the interplay between host genetics, the environment (diet in particular) and other factors, such as the gut microbiota. Accurate diagnosis and staging of NAFLD are of utmost importance, with histological examination the gold standard in diagnosis so far. However, novel noninvasive methods to diagnose liver disease are rapidly evolving. Upon diagnosis of NAFLD or NASH, appropriate management must be started. Importantly, NAFLD can be managed successfully with diet and lifestyle changes, but pharmacological intervention is warranted when these methods fail. Many challenges lie ahead in the NAFLD field. NAFLD is a global problem, and, ultimately, from a societal perspective, it will be essential to attack the root cause of NAFLD to reduce the burden of diseases related to caloric excess and disordered metabolism. This goal will require a broad effort of all stakeholders to address the social, economic, cultural, and medical underpinning of obesity and its related conditions, including NAFLD.

The aim of this book is to provide a comprehensive review of the present standing of NAFLD. I wish to thank the Authors, whose brilliant work has been of utmost importance for the current understanding of this disease, for sharing their knowledge in this book.

Turin, Italy — Elisabetta Bugianesi

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Obesity and NAFLD: Same Problem?

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Lucia Brodosi, Francesca Alessandra Barbanti, Maria Letizia Petroni, Francesca Marchignoli, and Giulio Marchesini

1.1 Obesity, Lipotoxicity, and the Metabolic Syndrome

1.1.1 Pathophysiology

Obesity, i.e., accumulation of body fat, stems from positive energy balance, independently of the absolute amount of calorie intake and energy expenditure via physical activity. According to Unger hypothesis [1], adipocytes were specifically developed to protect organs and tissues during periods of overnutrition, also providing reserve for periods of undernutrition. To satisfy these needs, the adipocytes turned into a versatile endocrine gland, able to regulate food intake via leptin, acting on hypothalamus. A second hormone, adiponectin, counterbalances the action of leptin and is reduced in obesity [2]. Mutation in leptin and leptin receptors genes and changes in leptin and adiponectin levels might regulate fat accumulation, but unhealthy lifestyle probably remains the most relevant factor responsible for increased total body fat. Under these conditions, fatty acid recirculation may exceed the anti-steatotic potential of adipose tissue, and lipotoxic disease develops, characterized by fatty infiltration of non-adipose organs and tissues, including the liver. The secretion of pro-inflammatory cytokines and pro-oxidant substances by adipose tissue favors insulin resistance on glucose and lipid metabolism, leading to a cluster of metabolic changes grouped to define the metabolic syndrome (MetSyn). The definition of MetSyn changed in the course of the years; obesity per se has never been considered a mandatory feature, but waist circumference (a surrogate marker of visceral obesity) was always included and the cutoffs, also related to gender and ethnic differences, were progressively reduced to include cases classified in the

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overweight range by body mass index (BMI). Alberti and Zimmet first proposed enlarged waist circumference as mandatory feature [3], and the proposal was followed by the International Diabetes Federation [4] and is now widely accepted. In a pivotal study based on statistical analysis of factors associated with the so-called insulin-resistance syndrome, Maison et al. identified BMI and waist-to-hip ratio (a surrogate marker of visceral obesity) as the core components of MetSyn, supporting recent classifications [5]. However, many more metabolic alterations stem from insulin resistance, which have never been included in the definition (Fig. 1.1). The sequence of events starting from liver fat accumulation (steatosis) to hepatic necro-inflammation with/without fibrosis (steatohepatitis) to cirrhosis, when unrelated to alcohol abuse, constitutes another nominated but not elected component of MetSyn (nonalcoholic fatty liver disease—NAFLD). NAFLD is one of the most prevalent liver diseases worldwide, occurring in all countries and all ethnic groups [6], largely associated with obesity and other components of MetSyn [7, 8]. Fatty liver may also occur in normal-weight individuals (approx. 10–15% of total cases) [9], but it is much more prevalent in overweight/obese people and also in these cases liver fat positively correlates with insulin resistance [10, 11]: accordingly, nonalcoholic NAFLD and its progressive states (nonalcoholic steatohepatitis—NASH—and NASH-cirrhosis) may be considered the hepatic manifestation of MetSyn [12]. The

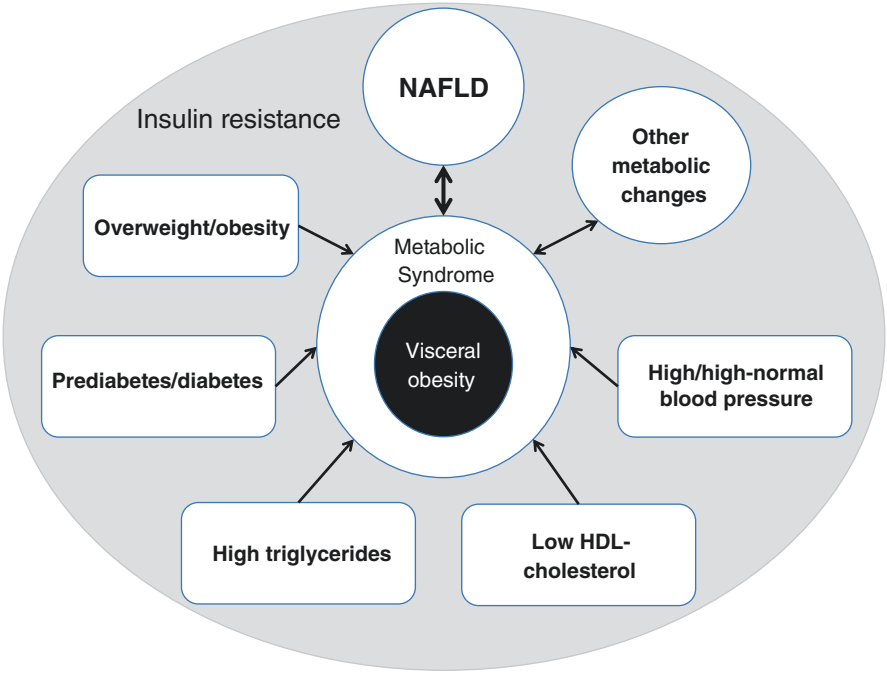

Fig. 1.1 Representation of the metabolic syndrome, having visceral obesity as the core components, and its relationship with NAFLD. Note the possible interdependence of NAFLD and metabolic changes, pointing at a causal and reverse causal association

association of NAFLD with MetSyn is so strict that several critical editorials have suggested that a new name should be given to NAFLD, to better highlight its pathogenic role [13–15]. This would achieve two main goals: (a) to identify the etiology in a positive way, avoiding the negative definition of "nonalcoholic"; (b) to consider the metabolic involvement as a possible comorbid condition of other liver diseases (namely alcoholic or viral liver disease). A proposal has recently been made to rename NAFLD as MAFLD (metabolic associated fatty liver disease), and it has immediately gained a wide consensus [16].

Although hepatic steatosis (pure fatty liver, without necroinflammation and fibrosis: i.e., nonalcoholic fatty liver—NAFL) is regarded as a benign stage, it may also progress to NASH in a subgroup of patients, and progression is difficult to forecast [17]. Visceral obesity is probably the main risk factor for NAFLD progression and inappropriate storage of triglycerides in adipocytes and higher concentrations of free fatty acids may add to increased hepatic lipid storage, insulin resistance, and progressive liver damage [18].

This is the general background linking whole-body fat (obesity) to hepatic fat accumulation (NAFLD), where four issues remain unsolved: (a) do obesity and NAFLD stem from a similar genetic background and similar lifestyles?; (b) do they coexist by simple association or is there a cause/effect relationship and, in this case, which comes first?; (c) have they a similar outcome and similar treatment?; and finally (d) does the existence of "lean NAFLD" challenge the pivotal role of adipose tissue accumulation?

In the following sections we will address these questions, in order to answer the title question.

1.1.1.1 Do Obesity and NAFLD Stem from Similar Lifestyles and a Similar Genetic Background?

The relationship between NAFLD and obesity is largely driven by similar pathogenic factors. Obesity is a complex disease, occurring from both genetic and lifestyle promoters (Fig. 1.2).

Dietary Factors

The present epidemic of obesity is largely dependent on excessive calorie intake and sedentary lifestyles, and at any stage of life obesity and NAFLD remain systematically associated (Fig. 1.2); similarly, there is considerable interest on calorie intake and dietary components in the development of NAFLD. In the presence of unhealthy lifestyles and behavioral factors, leading to enlarged adipose tissue and insulin resistance (IR), both lipolysis and de novo lipogenesis are expected to increase the risk of hepatic lipid depots, in association with high calorie (either high-fat or high-carbohydrate) diets [19]. Conflicting results have been reported on the dietary composition of patients with NALFD. In general, calorie intake did not differ between NAFLD and control subjects [20, 21], but macronutrient composition may differ. Studies using food frequency questionnaires (FFQs) reported a higher-than-normal habitual dietary fat intake [22], but in other cohorts higher carbohydrate intake and no differences in dietary fats were reported [21]. Notably, different dietary fats have

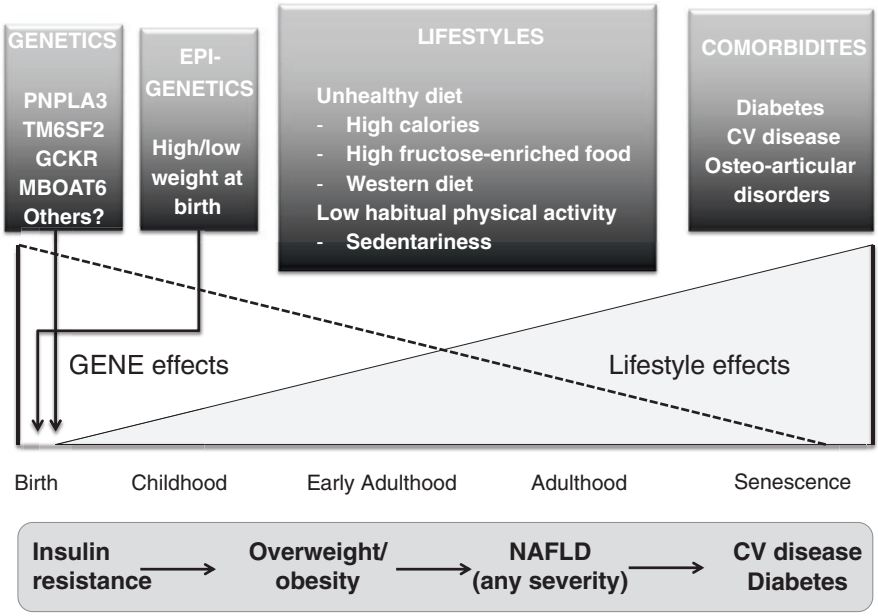

Fig. 1.2 Representation of the effects of genes, epigenetics, and lifestyles in the course of the years on the relative risk of NAFLD, NAFLD progression, and associated diseases. The relative importance of genes and epigenetic modifications is particularly high in infancy, whereas the importance of unhealthy lifestyles (both unhealthy diet and scarce physical activity) leading to obesity and NAFLD grows along the years

different effects on liver fat: diets rich in monounsaturated fatty acids (MUFAs) fat or n-6 polyunsaturated fatty acids (n-6 PUFAs) tend to reduce liver fat [23], whereas a high intake of saturated FAs increases liver fat more than a similar amount of n-6 PUFA [24].

Fructose-rich foods are the prototype of an unhealthy diet [25]. Fructose is largely metabolized in the liver, and fuels *de novo* lipogenesis, favoring steatosis [26]. Fructose is used to enrich sweetened beverages and processed foods, and its consumption is associated with a higher risk of obesity, as well as NAFLD detected by ultrasonography or magnetic resonance imaging [27, 28]. The deleterious effect of fructose might be specifically related to industrial fructose from processed foods and beverages, with limited effect of fruit fructose, when consumed with the several healthy nutrients also present in fruit, sharing antioxidant properties. This would explain the dichotomy between the risk associated with fructose and the beneficial effects of the Mediterranean diet, suggested to reduce the risk of NAFLD and NAFLD progression [29].

Also physical activity regulates triglyceride turnover and, indirectly, liver fat. Physical activity is also intimately associated with obesity, but its association with liver fat is independent of weight gain/weight loss. Any type (aerobic vs. resistance) [30], volume (time spent in exercise), and intensity (from low- to moderate- to

high-intensity) of physical activity, including leisure time and non-exercise activity, are important to decrease liver fat accumulation, compared with the time spent sedentarily, an additional risk factor for both obesity and NAFLD [31, 32].

Genetic Predisposition

A lot of data support a primary role of genetic factors shared between obesity and NAFLD (Fig. 1.2). Several genes have been reported to favor whole-body fat accumulation, although they do not completely account for obesity but should always be considered as cofactors interacting with unhealthy lifestyles [33]. Adiponutrin (PNPLA3) is an adipocyte protein with both lipolytic and lipogenic properties, regulated by insulin [34]. Gene polymorphism of the wild-type allele has been consistently associated with obesity, and in 2008, Romeo et al., in a genome-wide analysis of a large population of differing ethnic origin, identified a PNPLA3 allele strongly associated with both increased hepatic fat levels and hepatic inflammation. Notably, subjects homozygotes for the genetic variant had a much higher hepatic fat content and susceptibility to NAFLD [35]. The variant promotes hepatic injury, independently of insulin resistance and BMI [36], is also associated with higher risk of disease progression to advanced fibrosis and cirrhosis [37, 38], and confers a higher susceptibility to hepatocellular carcinoma [39].

Kozlitina et al. identified another variant in TM6SF2 rs58542926, a gene on chromosome 19, also associated with hepatic lipid accumulation [40], and also this variant was shown to increase the risk of disease progression [41, 42]. These data led the European Association for the Study of the Liver (EASL), together with the sister Associations of Diabetes and Obesity, to discuss the opportunity to include these two variants in a comprehensive assessment of the risk for disease progression in their joint NAFLD clinical practice guidelines [43]. Other variants may also modulate the risk (MBOAT7, GCKR, and MERTK), and one variant (a protein-truncating HSD17B13 variant) appears to be associated with a reduced NAFLD risk [44], opening a new frontier to disease prevention, via identification of subjects at higher risk.

According to Barker's hypothesis of fetal and infant origin of adult disease [45], epigenetic should also be considered. Epigenetic modifications are stable changes in the expression of DNA promoted by environmental risk factors in parents or in the intrauterine environment. The risk of NAFLD is not only associated with high BMI at birth [46] but also with low birth weight for gestational age [47]. Whether this reflects a more rapid catch-up following intrauterine retardation or a profound alteration in metabolic processes remains to be determined. The close relation with insulin resistance supports epigenetic regulation as the main driver [48]. In a comprehensive analysis of genetic predisposition, present and childhood demographic, metabolic and lifestyle variables, also including birth weight, the importance of in utero epigenetic modifications was extensively demonstrated [46].

As long as genetics remains a non-modifiable risk factor, lifestyle modifications, including diet and physical activity, targeting visceral adiposity remain the standard of care for patients with NAFLD and MetSyn. The health-care systems and hepatology communities need to implement measures aimed at reducing their causes; in the

area of NAFLD, child and adult obesity are a priority to reduce the burden of liver disease [49].

1.1.1.2 Do Obesity and NAFLD Coexist by Simple Association or Is There a Cause/Effect Relationship and, in this Case, Which Comes First?

Both childhood NAFLD and adult NAFLD are definitely more common in children and adults with obesity, respectively; long-term obesity might thus favor NAFLD progression to more severe stages, including liver failure and hepatocellular carcinoma, but the initial sequence of events remains difficult to determine. In a seminal study, Suzuki et al. tested the temporal occurrence of the various features of MetSyn in a cohort of subjects undergoing repeated screening in a Japanese workplace, free of any insulin resistance-related conditions, where elevated aminotransferase was assumed as surrogate biomarkers for NAFLD [50]. According to their analysis, weight gain and hypertriglyceridemia preceded NAFLD, whereas hypertension and altered glucose metabolism occurred later. Notably, weight gain and weight loss were consistently associated with altered and normal liver enzymes, respectively. These data were confirmed in a different cohort where incident fatty liver at ultrasounds was associated with the risk of incident hypertension [51] as well as incident diabetes [52].

Also epidemiological data and modeling studies support these findings. In Italy, the prevalence of NAFLD increases systematically along with obesity rates, with a time lag of approximately 5 years [8], and it is a significant risk factor for the future development of type 2 diabetes [53]. These data have been reproduced in different countries and different ethnic groups, and suggest that the future burden of NASH-cirrhosis might be extremely challenging for health-care systems [54].

A few long-term cohort studies are also available to support the role of weight gain on NAFLD and its long-term consequences. In apparently healthy individuals with no history of alcohol abuse, weight gain, and weight loss were associated with NAFLD incidence and remission, respectively, in a 7-year follow-up [55], and in a large cohort of normal-weight Korean individuals also a modest 2-kg weight gain was associated with the development of ultrasonographically detected fatty liver in a 5-year follow-up [56]. In a cohort of 44,248 Swedish men (18–20 years) enrolled into military service in their teens between 1969 and 1970, the risk of severe liver disease (i.e., diagnosis of decompensated liver disease, cirrhosis, or liver-related death) was associated with BMI and overweight in a follow-up of nearly 40 years [57]. The longer is the obesity status, the higher is the risk of NAFLD and its long-term consequences. Suomela et al. identified enlarged waist circumference, high body mass index (BMI), and sedentary lifestyles among the main drivers of future NAFLD, measured by ultrasonography in middle-aged adults [46]. In a Danish study of 285,884 schoolboys and girls, followed for over 30 years, the risk of primary liver cancer was increased by 20–30% in the presence of overweight/obesity at ages 7–13 [58].

By contrast, weight loss induced by lifestyle changes is significantly associated with improved liver function in cirrhosis. In the presence of obesity, an intensive

program coupling hypocaloric diet with supervised physical activity significantly reduced measures of portal hypertension by an extent dependent on weight loss in subjects with cirrhosis (24% with NAFLD) [59].

1.1.1.3 Have Obesity and NAFLD a Similar Clinical Outcome and Similar Treatment?

The burden of obesity per se on cardiovascular risk, chronic kidney disease (CKD), and cancer is well-known, and significantly impacts on life expectancy. Cardiovascular disease at age 60 reduces life expectancy by 6–10 years, but when coupled with metabolic diseases (cardiometabolic multimorbidity) life expectancy is reduced by 15 years [60]. The Emerging Risk Factors Collaboration group recently reported the effects of cardiometabolic multimorbidity, defined by the simultaneous coexistence of more than one conditions among type 2 diabetes, coronary heart disease and stroke, in adults who were overweight and obese compared with subjects with healthy weight. In over 120,000 adults, stratified according to BMI and without risk factors at baseline, and a mean follow-up of 10.7 years, the risk of developing cardiometabolic multimorbidity doubled in overweight individuals (odds ratio [OR] 2.0, 95% CI 1.7–2.4), and further increased to 4.5 (3.5–5.8) in type 1 obesity, and to 14.5 (10.1–21.0) in subjects with obesity class II–III. The association was maintained irrespective of gender, socio-economic status, age, and lifestyles [61]. This study highlights the importance of obesity, when coupled with other metabolic diseases, i.e., of MetSyn in deadly outcomes.

The association of obesity with CKD is also well demonstrated. Hsu et al. identified obesity as a risk factor for end-stage kidney disease in 2006 [62] and again metabolic multimorbidity significantly increases the risk. In subjects with and without MetSyn, both overweight and obesity more than double the risk of CKD [63], but CKD is also a correlate of cardiovascular morbidity, further increasing the burden of disease [64].

The most intriguing association of obesity is the risk of cancer, largely ignored by patients and scarcely perceived by health professionals. The most impressive data came from the seminal study of Calle et al. [65], in a prospective study of more than 900,000 adults, free of cancer at enrollment. During a follow-up of 16 years, the risk of death from cancer (any site) was increased by more than 50% in individuals with obesity, with particular risks for specific sites (including the liver). These data have been repeatedly confirmed in different settings and different ethnic groups [66]; notably, long-term weight loss induced by bariatric surgery not only increases life expectancy, but initial data are accumulating on its role in reducing the risk of incident cancer [67]. Of note, the cancer risk associated with obesity might be directly driven by liver fat [68], with NAFLD as the main predictor of future extrahepatic cancer also in obese individuals [69].

How much do the same factors dictate the outcome of NAFLD? Although NAFLD may progress to NASH-cirrhosis and end-stage liver disease remains a dreadful outcome, the majority of cases have a cardiovascular outcome. In a long-term follow-up study of a NAFLD cohort, Ekstedt et al. found an increased risk of cardiovascular death [70], although fatty liver was unable to predict cardiovascular

death in subjects with established coronary artery disease [71]. Similarly, NAFLD patients are at higher risk of hepatocellular cancer [72], also in the absence of cirrhosis. In an ultrasonography-defined NAFLD cohort followed by regular check-ups for over 7 years, the cancer incidence rate was significantly increased (hazard ratio [HR] 1.32; 95% confidence interval [CI] 1.17–1.49) [73]. After adjustment for demographic and metabolic factors, three cancers were significantly associated with NAFLD: hepatocellular carcinoma (HR 16.7; 95% CI 2.1–133.8), colorectal cancer in males and breast cancer in females, i.e., cancers significantly associated with obesity, independently of fatty liver.

Weight loss is the standard treatment of both obesity and NAFLD. These issues will be dealt with in another chapter; suffice here to say that weight loss, both achieved by lifestyle changes or by bariatric surgery simultaneously decreases the burden of both obesity and NAFLD and NAFLD progression to more advanced stages of the disease.

1.1.1.4 Does the Existence of "Lean NAFLD" Challenge the Pivotal Role of Adipose Tissue Accumulation?

Since the original identification of NAFLD as a specific condition associated with MetSyn, it became apparent that a variable proportion of cases was not associated with obesity. These cases, identified as "lean NAFLD," account for 10–15% of total NAFLD individuals in different cohorts, depending on age, gender, and particularly on the clinical setting [9]. In most cases they are by no means lean, but have a limited amount of body fat, fulfill the criteria for normal weight or overweight, but frequently have an excess of visceral fat (visceral obesity) [6]. They might represent a variant of the so-called "metabolically obese, normal weight" phenotype [74], described in at least 5% of the population in Western countries. This subgroup, lying on the opposite end of "metabolically healthy obese" population along a spectrum dictated by genes, diet, physical activity, and inflammation [74], comprises individuals who are non-obese, frequently sedentary, and who have impaired insulin sensitivity, increased cardiovascular risk and increased liver lipid levels as the consequence of a decreased capacity of fat-storing cells [75]. When compared with individuals with overweight or obese NAFLD, these subjects are usually younger, are nonetheless insulin resistant, and have higher plasma triglyceride levels, possible expression of a more severe alteration in lipid metabolism [76], but variable and sometimes more severe degree of necroinflammation and fibrosis [77]. In their most advanced stages, they are frequently identified as "cryptogenic cirrhosis", which has produced some debate in the interpretation of diagnostic tests and on the identification of NASH-cirrhosis and cryptogenic cirrhosis as different entities [78, 79].

The histologic and clinical outcome of lean NAFLD has attracted a lot of attention. The largest series of "lean NAFLD" comes from studies carried out in Eastern countries, which are at higher risk of insulin resistance for minimal visceral adiposity, as demonstrated by the specific cutoffs of waist circumference for MetSyn dictated by International agencies [4]. In a systematic review with meta-analysis, Sookoian et al. compared the histological outcomes of lean NAFLD series ($n = 493$ individuals) with overweight/obese NAFLD individuals [80]. Contrary to initial

findings, the authors concluded that lean NAFLD is characterized by less severe histological features as compared to overweight/obese NAFLD [80]. Also disease progression has never been clearly defined. In the study of Fracanzani et al., the risk of cardiovascular-related events was not systematically different between lean and overweight/obese NAFLD during a follow-up of 49 months, but the numbers of deaths and events were too small to derive solid conclusions [77]. On the contrary, in a large multicenter analysis published only in abstract form, the death rate of lean NAFLD was reported to be higher compared to the event rate in non-lean individuals [81]. This occurred despite lean NAFLD being characterized by less severe disease, a low number of comorbidities and lower levels of liver enzymes.

In conclusion, lean NAFLD remains a scarcely defined condition, which partly blurs the relation between obesity and NAFLD. However, it might indeed represent the end of a large spectrum where different genetic and lifestyle factors interact to determine liver disease incidence and progression.

1.2 Conclusions

The accumulation of fat droplets in the hepatic parenchyma is driven by factors synergistically acting to increase triglyceride flow to the liver (diet and metabolic factors, endotoxemia from gut microbiota, genetic factors). They are shared between obesity and NAFLD, as are the levels of adipokynes, both leptin and adiponectin, that are putative mediators of lipotoxicity [2].

A large body of evidence supports the concept that NAFLD rarely dissociates from obesity, and in these cases visceral fat accumulation is nonetheless present, also accounting for NAFLD progression to fibrosis and cirrhosis, as well as to T2DM and other metabolic abnormalities. The best evidence comes from intervention studies, showing that body weight loss, whatever the strategy used to reduce obesity (lifestyle changes, low-calorie diet, physical activity, bariatric surgery), remains the most effective way to reduce the incidence and prevalence of NAFLD in selected cohorts and in the general population, its progression to cirrhosis, and liver disease-related morbidity and progression also in the presence of cirrhosis [59]. An expert report, recently released from the European Association for the Study of the Liver focusing on the burden of liver disease in Europe (HEPAHEALTH project), concludes that tackling obesity is the only way to reduce the burden of NAFLD, by combining health policies with food interventions at population level [49]. We need to develop new strategies to counsel, motivate, educate toward healthier lifestyles the high number of individuals at risk of advanced liver disease all over the world [49]. Web-based programs are at the forefront [82], and should be exploited considering the difficulties faced by Liver units in preparing adequate educational programs. However, their effectiveness is limited if not integrated with face-to-face visits and contacts with specialists trained in motivational interviewing, considering the scarce motivation from patients' side [83]. Interventions aimed at curbing the NAFLD epidemics are urgently needed not only to reduce the burden on National Health Systems but also to decrease the environmental impact and the

costs of the Western dietary model [84]. The Mediterranean diet is qualifying as a dietary pattern able to reduce the risk of obesity, NAFLD, and associated cardiovascular risk, also favoring a sustainable healthy eating behavior [85].

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The Burden of NAFLD Worldwide

2

Zobair Younossi and Linda Henry

2.1 Background

Nonalcoholic fatty liver disease (NAFLD) is rapidly becoming the most common cause of chronic liver disease and is now among the top causes of cirrhosis, hepatocellular carcinoma (HCC), and indications for liver transplantation in United States and probably the rest of the world [1, 2]. However, NAFLD is not a single disease but rather a spectrum of clinico-pathologic liver diseases that include nonalcoholic fatty liver (NAFL or simple steatosis), nonalcoholic steatohepatitis (NASH), cirrhosis, and its complications [3]. Furthermore, NAFLD is considered the hepatic manifestation of metabolic syndrome since most NAFLD patients have visceral adiposity, insulin resistance, and/or type 2 diabetes mellitus, hypertension, hypercholesteremia, and hypertriglycemia. In fact, the more components of metabolic syndrome are present in patients with NAFLD, the higher the risk of advanced fibrosis and liver-related mortality [1–4].

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2.2 Global and Regional Prevalence of NAFLD and NASH

Currently, 25% of the world is thought to have NAFLD with the highest prevalence being reported from the Middle East and South America (31.79% and 30.45%, respectively) and the lowest from Africa (13.48%) [4]. The prevalence of NAFLD in North America, Europe, and Asia has been reported as 24.13%, 23.71%, and 27.37%, respectively. Other histologic-based studies from Europe have suggested a NAFLD prevalence of approximately 20% while in Asia NAFLD prevalence is thought to range from 19 to 23% [3, 4].

Among patients with type 2 diabetes, the prevalence of NAFLD is higher at 57.80% [5]. Furthermore, among the morbidly obese, NAFLD prevalence has been found to be 95% [6].

Since diagnosis of NASH is based on histology, true prevalence rates for NASH in the general population is not known. On the other hand, estimated prevalence rates for NASH in the general population is considered to range from 1.5 to 6.45% [4]. In contrast, the prevalence of NASH in patients with type 2 diabetes is higher. A recent meta-analysis suggested that the overall prevalence of NASH among biopsied diabetics is 65.26% with 15.05% of these patients having advanced fibrosis (fibrosis $\geq$ F3) [5]. Finally, the prevalence of NAFLD among very obese individuals undergoing bariatric surgery is over 95%, while 20–50% of them have NASH and about 10% have advanced fibrosis [6].

As the rates of obesity, type 2 diabetes mellitus, and insulin resistance increase among an aging population, so does the prevalence of NAFLD. Several recent global modeling analyses based on changes in adult obesity and DM in the United States have determined that the prevalence of NAFLD is set to grow exponentially over the next decade [7–9]. Similar rates are being reported from other regions of the world. In Saudi Arabia, there is projected to be 12,534,000 NAFLD cases, while for the United Arab Emirates there are projected to be approximately 372,000 cases [9]. As a result, the prevalent cases of compensated cirrhosis and advanced liver disease are projected to at least double by 2030, while an annual liver-related mortality is projected to be an annual 4800 deaths in Saudi Arabia and 140 deaths in UAE [9]. When modeling NAFLD for Asia and Europe, researchers determined that dependent on the rate of increase for obesity and diabetes, there could be a 0–30% increase in total NAFLD cases between 2016 and 2030 [8]. Due to urbanization, China is expected to experience the highest increase in NAFLD cases, incident cases of cirrhosis, decompensated cirrhosis, hepatocellular carcinoma, and death. Within the European countries, Germany is expected to experience the highest increase in NASH and HCC cases by 2030, while France is projected to have the most cases of compensated and decompensated cirrhosis by 2030 [8].

2.3 Natural History of NAFLD

The data about the natural history studies of NAFLD comes from several lines of evidence. Initial studies were histologic cohorts from tertiary care centers with mortality data [9–11]. Most of these studies and their meta-analytic summaries suggested that patients with histologic NASH were predominantly progressive [12–14]. Subgroup analysis suggested that presence of type 2 diabetes and advanced histologic fibrosis at baseline predicted mortality [15, 16]. The second line of evidence also comes from tertiary care centers where repeated liver biopsies were performed during clinical follow-ups [17, 18]. These studies also suggested that patients with NASH can show progression of fibrosis [17, 18]. Furthermore, they observed that some patients whose initial liver biopsies were not consistent with NASH also progressed [17, 18]. Furthermore, some patients showed regression of fibrosis regardless of the initial histology [1, 18]. The third line of evidence comes from placebo arms of clinical trials of NASH with sequential protocol biopsies [19]. These data provide a much more dynamic picture. In fact, some patients with NASH and fibrosis progress while others regress [19]. The exact reasons for these fluctuating patterns of progression and regressions are not known. The latest evidence supporting the progressiveness of NAFLD is related to the observation that most patients with cryptogenic cirrhosis have the profile of patients with NASH and have a high recurrence of NASH post liver transplantation [20, 21]. Although this has created some controversy [22], a recent biopsy-based data confirmed that most of these patients do have the clinical profile of patients with NAFLD [23].

Although these data suggest that the exact course of a patient with NAFLD can vary and fluctuate, one can draw some generality about the natural history of NAFLD. In this context, the histologic subtype of NAFLD that can be classified as NASH is associated with highest risk for progressive liver disease [17, 18]. In fact, the risk becomes higher in those with significant hepatic fibrosis [12, 24–27]. In this context about 20–30% of patients with NAFLD will have NASH and of these 10–15% can progress to cirrhosis. As noted previously, those with increasing number of metabolic comorbidities, especially type 2 diabetes mellitus, are at the highest risk of progression [3, 5, 12, 26]. In contrast, those with early NASH and individuals with non-NASH NAFLD more commonly die of cardiovascular causes and possibly non-HCC cancers [12–15, 27, 28] (Fig. 2.1).

The time line of progression can vary due to underlying risk factors. In general, the average progression from one disease state to another can take up 7.7 years [26]. In studies using paired liver biopsies, researchers found that 30% of patients with NAFL and NASH had progressive fibrosis while 20% had NAFLD regression over 2.2–13.8 years [27]. Nevertheless, some patients may experience faster progression

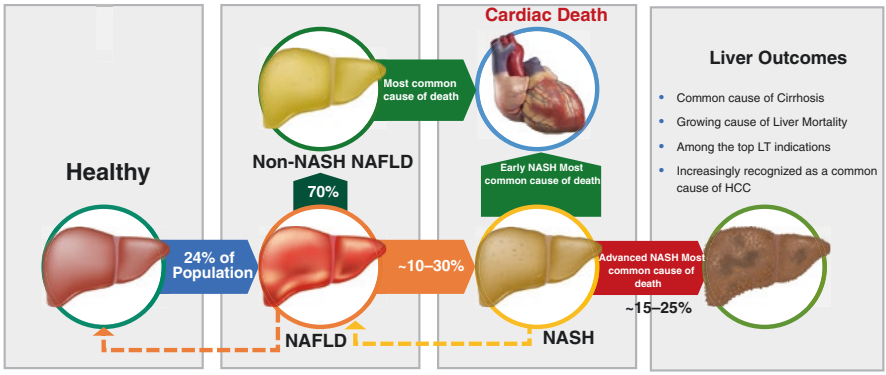

Fig. 2.1 The natural history of NAFLD

rates, especially those with visceral obesity, type 2 diabetes, older age, and possibly Hispanic ethnicity [17, 18, 28]. This last factor may be different based on the country of origin and may be affected by PNALP3 genetic predisposition [29, 30].

2.4 Hepatic Complications of NAFLD

Despite the complexity of the natural history of NAFLD, there is increasing evidence that NAFLD is becoming the most common cause of liver disease [31]. Additionally, data from the United States indicates that NASH-related cirrhosis has doubled over a decade or so [32]. Furthermore, the risk of hepatocellular carcinoma (HCC) related to NAFLD in the United States has increased substantially. In fact, the incidence of HCC among NAFLD patients is estimated to be 0.44 per 1000 person years [17, 18]. In addition, patients with NAFLD fibrosis stages 3 and 4 have almost a seven times higher risk of developing HCC compared to those without significant liver disease [17, 18, 26, 27, 33, 34]. It is interesting to note that presence of metabolic syndrome especially obesity and insulin resistance may hasten development of HCC [35–37].

Obviously, development of cirrhosis, its complications, and HCC lead to increased risk of liver-related mortality. Liver-specific mortality among those with NAFLD has also been reported to be 0.77 per 1000 person years [38, 39]. This rate is almost 10 times higher in patients who develop NASH with a reported rate of 11.77 per 1000 person years [38, 39]. Similarly, the overall mortality per 1000 person years was reported to be 15.44 for those with NAFLD and 25.56 for those with NASH, whereas others have reported that patients with fibrosis stages 3 and 4 had an overall mortality risk three times greater than those without liver disease [34, 38]. An important issue related to NASH-HCC was observed by Surveillance, Epidemiology, and End Results Database (SEERS) study which suggested that, although NAFLD was among the top three causes of HCC, those with NAFLD HCC incurred a higher mortality at 1 year post-diagnosis [35].

As noted previously, for the entire cohort of individuals with NAFLD, cardiovascular mortality remains the most common cause of death [40]. In this context, liver-related death is the third cause of death after cardiac and cancer-related deaths [9]. On the other hand, for patients with NASH and advanced fibrosis, liver-related mortality predominates [23]. Although NAFLD and cardiovascular diseases (CVD) share common comorbidities, the exact reason for high prevalence of CVD in NAFLD patients is not known. Some investigators believe that endothelial dysfunction in patients with NAFLD may be contributing to the increased risk of cardiovascular mortality, but the exact mechanisms have not been clarified [40].

In addition to mortality, listing for liver transplantation is an important outcome for patients with liver disease. In this context, NAFLD/NASH is rapidly becoming a major indication for liver transplantation in the United States [2, 41]. A recent analysis of the United States Scientific Registry of Transplant Recipients (SRTR) from 2012 to 2016 found that NASH was the fastest increasing indication for liver transplantation among those listed positioning NASH to become the most common cause for liver transplantation in the near future [41]. Another analysis of SRTR suggests that NASH-related is the fasting growing indication for HCC listing for liver transplantation in the United States [1]. Given the lack of systematic screening or failure of screening for HCC in these individuals, it is possible that most cases of NASH-related HCC do not get listed for liver transplantation or die while waiting for an organ [42, 43].

2.5 Differences in Outcomes of Western NAFLD from Eastern NAFLD

Although most patients with NAFLD are obese, some are considered lean [44–50]. As previously noted, prevalence of lean NAFLD in the United States is about 7% [50]. In contrast, these rates are higher in some Asian countries. Using region-specific BMI thresholds, the prevalence of lean NAFLD has been reported to be 20% in India, 15.2% in Japan, 15% in China, 12% in Greece, 12.6% South Korea, and Iceland [44–49].

Although lean NAFLD is metabolically less abnormal than obese and overweight NAFLD, they still have higher rates of insulin resistance and diabetes than lean controls without NAFLD [50]. In a study of lean Korean NAFLD patients, there was an increased prevalence of hypertriglyceridemia, hyperuricemia, insulin resistance, and central obesity but a lower prevalence of diabetes, hypertension, hypertriglyceridemia, low-HDL cholesterol, central obesity, and metabolic syndrome than in other studies of lean NAFLD [48]. Others have found both lean and obese NAFLD have excess abdominal adipose tissue. On the other hand, the exact mechanism by which the genetic and environment factors influence progression of NAFLD lean individuals needs further study [51, 52]. Others have reported using data from a large multicenter, biopsy-proven cohort was that there was an increased overall mortality rate in lean patients compared to those that are overweight or obese with NAFLD

[53]. On the other hand, another study also using biopsy-proven NAFLD over a long-term follow-up of a median of 19.9 years (range 0.4–40 years) representing 12,631 person years found that patients with lean NAFLD were at a higher risk for development of severe liver disease compared to patients with NAFLD and a higher BMI [54]. This finding is especially important as it has been recently found that it is the stage of fibrosis not the presence of NASH that predicts mortality and time to development of severe liver disease [25, 55]. Although still controversial, this study suggests that lean NAFLD patients may have a more aggressive course of NAFLD that may require more close surveillance. However, further study is warranted to determine appropriate timing of surveillance especially as the most accurate non-invasive method to diagnosis NAFLD is still under debate [56].

2.6 Economic Impact of NAFLD and NASH

The economic burden of NAFLD/NASH has recently been assessed using different methodology and projected to be immense [1, 7, 8]. A Markov-based model estimated that in the United States, there are over 64 million people with NAFLD accounting for an annual direct medical cost of about $103 billion ($1613 per patient). Among the four European countries, approximately 52 million people were estimated to have NAFLD with an annual cost of about €35 billion (from €354 to €1163 per patient), while the costs, prevalence, and incident rates were found to be the highest in patients aged 45–65 regardless of the country of origin [57].

In a separate Markov model focusing on NASH and advanced NASH in the USA, investigators determine that, in 2017, there were 5,527,812 adult subjects with NASH in the United States. The life time cost burden of all NASH patients in the United States was estimated to be $82,704,934,702, while the cost of those with advanced NASH was $31,526,708,220. The projections of costs for each age-specific NASH cohort have the potential to increase about 400% in the next 5 years [58].

In addition to the decision analytic models, administrative billing databases can also provide some data about resource utilization related to NAFLD and NASH. In the United States, a number of studies have used the Medicare database (a federally sponsored insurance provided to all citizens 65 years and older and others who meet certain criteria) for resource utilization estimations related to different liver diseases. One such study for patients with NAFLD, investigators found the mean yearly inflation-adjusted outpatient charges for Medicare patients with NAFLD doubled from 2005 to 2010 ($2624–$3308 in 2005 to $3608–$5132) [59]. An outpatient and inpatient follow-up study also using the Medicare database confirmed the enormous impact of NAFLD when investigators reported that the median total hospital charge for NAFLD patients was $36,289 in 2010 and increased with disease severity [60].

2.7 Conclusions

In summary, the prevalence and incidence of NAFLD is growing globally. NAFLD is not a benign disease as it can progress to advanced liver disease, hepatocellular carcinoma, liver transplantation, and death. Though the number of patients who actually progress is small on a global level, the burden is substantial. In addition to the clinical burden of NAFLD also carries a tremendous economic burden which is likely to increase as the population continues to age. Continuous study is needed to develop interventions to reverse the course of NAFLD especially as our understanding of NAFLD evolves.

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Histopathology of Nonalcoholic Fatty Liver Disease

3

Dina G. Tiniakos and Stratigoula Sakellariou

3.1 Introduction

Nonalcoholic fatty liver disease (NAFLD) is a complex metabolic disease, which is etiologically correlated with systemic and hepatic insulin resistance, in both adults and children. NAFLD is considered by many as the hepatic manifestation of the metabolic syndrome and is currently the most common chronic liver disease in the Western world, with a prevalence of 20–30% in adults. NAFLD is also recognized as a significant pediatric chronic liver disease correlated with the increase of childhood obesity all over the world [1].

The diagnosis of NAFLD is clinicopathological. The minimal histological change is hepatocyte steatosis, referring to the accumulation of triglycerides within hepatocytes in a patient who does not consume significant amounts of alcohol (women >14 and men >21 drinks per week) [2, 3]. NAFLD has a wide histological spectrum including simple steatosis, which generally has a benign course and is present in all patients, and nonalcoholic steatohepatitis (NASH), the progressive form of the disease, seen in 7–30% of NAFLD patients depending on the geographical area. Progressive fibrosis develops in 26–37% of NASH patients, while 9–20% become cirrhotic. Overall, 3–5% of NAFLD patients may end up with advanced liver disease with cirrhosis. Hepatocellular carcinoma (HCC) may develop in 4–12.5% of NASH cirrhosis patients, while it is now accepted that HCC can occur in pre-cirrhotic NASH [4, 5].

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Despite the progress in the development of noninvasive methods for NASH diagnosis and evaluation of fibrosis in NAFLD, liver biopsy remains the best and most accurate tool for confirming the presence or excluding NASH in patients with liver steatosis on imaging, appropriate clinical characteristics, mainly related to the metabolic syndrome, and unexplained hypertransaminasemia. However, currently, liver biopsy is performed only in selected NAFLD patients mainly because it is an invasive, painful, and costly procedure with rare, but existing, complications. In addition, in most patients the disease will run a benign course and since there is no approved pharmacological treatment for NASH, a liver biopsy may not alter patient management. Liver biopsy has indication for NAFLD patients with known risk factors for developing steatohepatitis with significant fibrosis, such as older age (>40 years), obesity, and/or diabetes type II. Current clinical guidelines recommend liver biopsy in patients with surrogate markers of fibrosis and/or transient or magnetic resonance elastography values indicative of medium to high risk of advanced fibrosis. Liver biopsy is also advised in patients with suspected NAFLD and competing etiological factors for hepatic steatosis or possible coexisting chronic liver disease [2, 3].

This chapter will focus on the histopathology of adult and pediatric NAFLD in the setting of the metabolic syndrome and will not refer to NAFLD in other clinical settings, such as drug- and toxin-induced fatty liver disease, allograft liver, total parenteral nutrition, nutritional disorders, and inherited metabolic diseases.

3.2 Histopathology of Adult NAFLD

3.2.1 Steatosis

Hepatic steatosis, as described above, refers to the deposition of lipids, mainly triglycerides in the cytoplasm of hepatocytes. Lipids within hepatocytes are stored in vesicles which, when large, displace the nucleus to the periphery of the affected cells. Normal liver may contain a few lipid-laden hepatocytes but currently the histological diagnosis of nonalcoholic fatty liver (NAFL) or simple steatosis is rendered if more than 5% of hepatocytes contain lipid droplets [4]. This arbitrary 5% cutoff value is based on early biochemical studies showing that approximately 5% of normal liver weight is composed of lipids [6].

On routine histology, fat accumulation corresponds to empty intracytoplasmic vacuoles since lipids dissolve during histological processing. Steatosis is described as macrovesicular, microvesicular, or mixed. Macrovesicular steatosis is characterized by either a single, large, fat droplet that almost replaces the cytoplasm and pushes the nucleus peripherally or by a few smaller fat droplets and a centrally placed nucleus (small droplet macrovesicular or mediovesicular steatosis) (Fig. 3.1). In microvesicular steatosis, the hepatocyte cytoplasm has a foamy appearance because it is filled with numerous tiny lipid droplets while the nucleus remains central. In NAFLD, steatosis is of macrovesicular or mixed type. In the latter, small non-zonal patches of microvesicular steatosis are encountered within larger areas

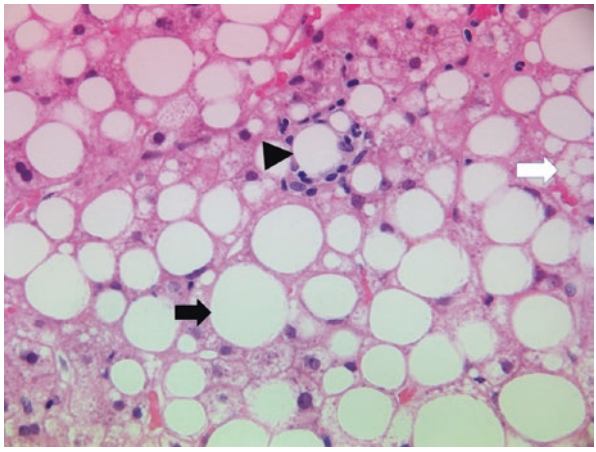

Fig. 3.1 Nonalcoholic fatty liver: Large (black arrow) and small droplet (white arrow) macrovesicular steatosis and a lipogranuloma (arrowhead). Hematoxylin–Eosin ×200

of macrovesicular steatosis [4, 7–9]. Microvesicular steatosis in NAFLD has been correlated with increasing severity of steatosis and presence of steatohepatitis [10]. Extensive microvesicular steatosis, similar to the "alcoholic foamy degeneration" sometimes seen in patients with severe alcohol-related liver disease (ALD) and associated with adverse prognosis [11], is not a feature of NAFLD.

In early NAFLD, the pattern of liver injury is centrilobular (zone 3 of the hepatic acinus) with steatosis first appearing around the terminal hepatic venule (THV) but as the disease progresses the entire lobule/acinus can be affected. Rarely, in obese adults and in some pediatric cases, steatosis shows a periportal (zone 1) predilection. With NAFLD progression, architectural remodeling due to fibrosis may result to a non-zonal pattern of steatosis. In the cirrhotic stage, steatosis may disappear altogether leaving no trace of fatty liver disease etiology [8]. It is not surprising, therefore, that NAFLD is considered responsible for 30–75% of cases of cryptogenic cirrhosis [5].

Pure steatosis is rare in NAFLD. Usually, a chronic mononuclear cell inflammatory infiltrate is present in the liver parenchyma, composed mainly of CD4-positive and CD8-positive T lymphocytes, rare plasmacytes, and monocytes [12]. Lobular inflammation in NAFLD can also be mixed with additional presence of neutrophil and occasional eosinophil polymorphs. Macrophages, isolated or in clusters of four–five (microgranulomas), may be encountered, while lipogranulomas, consisting of a steatotic hepatocyte or fat droplet surrounded by macrophages, mononuclear cells, and a rare eosinophil, are frequent in the lobules (Fig. 3.1). Macrophages are either liver-specific (Kupffer cells) or derive from circulating blood monocytes infiltrating the liver [13]. Portal tracts may show a mild chronic or mixed inflammatory infiltrate without interface activity [4, 7–9]. Inflammation is a driver for the development and progression of NAFLD [14]. The majority of NAFLD patients with pure steatosis or steatosis with inflammation will have a benign, non-progressive course. However, data from studies with paired liver biopsies at least 1 year apart have shown that both steatosis and steatosis with inflammation can progress to steatohepatitis with advanced fibrosis [15, 16].

Mild fibrosis, located either in portal tracts or in perivenular areas along sinusoids, may be detected in liver biopsies of adults with steatosis or steatosis with inflammation without evidence of hepatocyte ballooning. In these cases, fibrosis may be indicative of past active steatohepatitis since NAFLD is now accepted as a dynamic disease where activity may fluctuate over time. On the other hand, the absence of hallmarks of hepatocellular injury in fibrotic NAFLD may be due to sampling variability [9].

3.2.2 Steatohepatitis

Currently, the minimal histological criteria for the diagnosis of steatohepatitis are steatosis, lobular inflammation, and hepatocellular injury in the form of ballooning [4] (Fig. 3.2) with a centrilobular (zone 3) predilection (Fig. 3.3). Fibrosis is not a required feature for the diagnosis of NASH as it is not required for the diagnosis of chronic hepatitis of other etiology.

Hepatocyte ballooning is a key histological feature of NASH and together with lobular inflammation reflect disease activity. The pathogenesis of ballooned hepatocytes includes alterations of microtubules due to oxidative stress, loss of keratins 8 and 18 (K8/18), which form the intermediate filament cytoskeleton of hepatocytes, retention of fluid, modification of intracytoplasmic microvesicular fat, and endoplasmic reticulum dilatation [9]. Ballooned hepatocytes lose their polygonal shape and become rounded as a result of injury to their K8/18 cytoskeleton. They have a characteristic rarefied, reticulated, non-vacuolar cytoplasm with a centrally located nucleus and conspicuous nucleoli [4, 9, 12]. In their classical form, ballooned hepatocytes are 1.5–2 times larger compared to non-steatotic normal hepatocytes, but non-classical forms with normal size are recognized, retaining the round shape and characteristic cytoplasmic changes [17]. Sometimes, ballooned hepatocytes are encircled by neutrophils, in a lesion known as satellitosis, and delicate collagen fibrils (pericellular

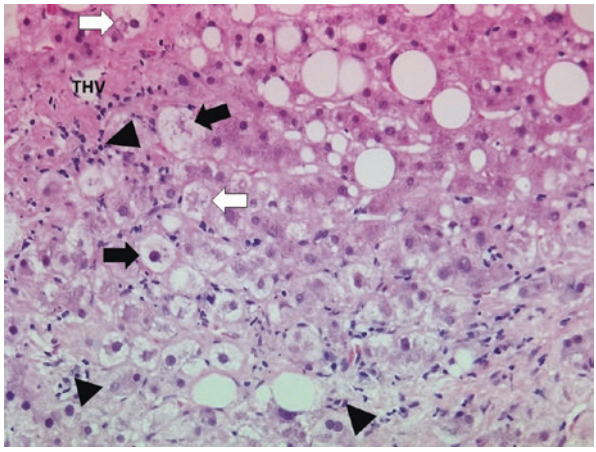

Fig. 3.2 Nonalcoholic steatohepatitis: Steatosis, inflammatory foci (arrowheads), and ballooned hepatocytes (black arrows), some including Mallory-Denk bodies (white arrow) in zone 3 close to the terminal hepatic venule (THV). Hematoxylin–Eosin ×200

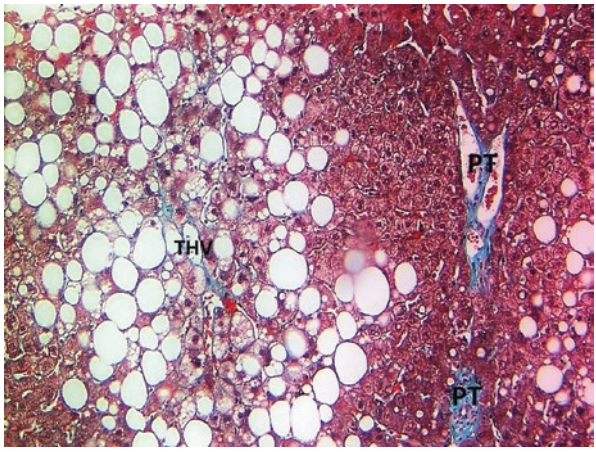

Fig. 3.3 Nonalcoholic steatohepatitis: Predilection of steatosis, lobular inflammation, hepatocyte injury, and sinusoidal fibrosis in zone 3 close to the terminal hepatic venule (THV). The periportal liver parenchyma shows minimal changes. Portal tract (PT). Masson trichrome stains collagen fibers blue, ×100

fibrosis) [4, 9, 12]. Recent studies have highlighted that in ballooned hepatocytes the procedure of cell death is initiated but not completed, intermediate filament proteins are degraded, the pro-fibrogenic Sonic hedgehog (Shh) pathway is activated, and ubiquitinated proteins are accumulated [13]. The inter-observer variability for the histological detection of ballooned hepatocytes is rather high, especially among general pathologists [18], therefore objective markers for their detection, such as loss of immunohistochemical expression of K8/18 [19] and increased expression of Shh [20], have been proposed to facilitate their recognition. The value of these ancillary immunostains for the diagnosis of NASH in routine practice remains to be confirmed. Shh immunohistochemical expression in ballooned hepatocytes has been correlated with NASH severity and progression [21]. Other forms of hepatocyte injury such as apoptosis and, less commonly, necrosis can also be observed in NASH. The number of apoptotic hepatocytes correlates with NASH severity [4].

Ballooned hepatocytes may occasionally contain Mallory-Denk bodies (MDB) (Fig. 3.2). There are cytoplasmic inclusions of hyaline eosinophilic material composed of insoluble ubiquitinated proteins, including K8/18, bound to the autophagy regulator protein p62 [22]. MDB are highlighted by immunohistochemistry for K8/18, ubiquitin, and p62, and their presence is associated with increased disease severity [12, 23]. MDB are not specific for NASH and are not essential for the histological diagnosis.

In most cases of NASH, lobular inflammation is mild to moderate and is chronic or mixed, as described in Sect. 3.2.1. Severe lobular infiltration by neutrophils and prominent satellitosis are not a feature of NASH, and if present, alcoholic etiology may be suspected [4]. Mild chronic portal tract inflammation is common in NASH, and its severity increases progressively in parallel with increasing steatohepatitic injury and fibrosis stage [24]. Increased portal inflammation is a feature of disease resolution in posttreatment liver biopsies in NASH clinical trials [4]. However, severe portal inflammation and/or more than mild, focal interface activity should always raise the suspicion of another or concurrent etiology for the chronic liver disease.

Similar to other chronic liver diseases, NASH is often accompanied by fibrosis that may progress to cirrhosis in some patients. In adult NASH, extracellular collagenous matrix (ECM) is initially deposited in the space of Disse along the sinusoids (sinusoidal or perisinusoidal fibrosis) and may surround hepatocytes (pericellular fibrosis) (Figs. 3.3 and 3.4a). The centrilobular areas (acinar zones 3) are the first to be affected and fibrosis progressively expands peripherally [4, 7–9]. Portal/periportal fibrosis develops as the disease evolves and, in more advanced stages, fibrous septa originating from the THV and possibly from portal tracts merge to form central–central, central–portal, and portal–portal collagenous bridges (bridging fibrosis). Further architectural remodeling results in cirrhosis with nodular areas of regenerating hepatocytes completely surrounded by fibrous septa (Fig. 3.4b) [4, 7–9]. When the characteristic sinusoidal/pericellular fibrotic pattern is still discernible, NASH cirrhosis can be diagnosed in the appropriate clinical setting even in the absence of steatosis [7].

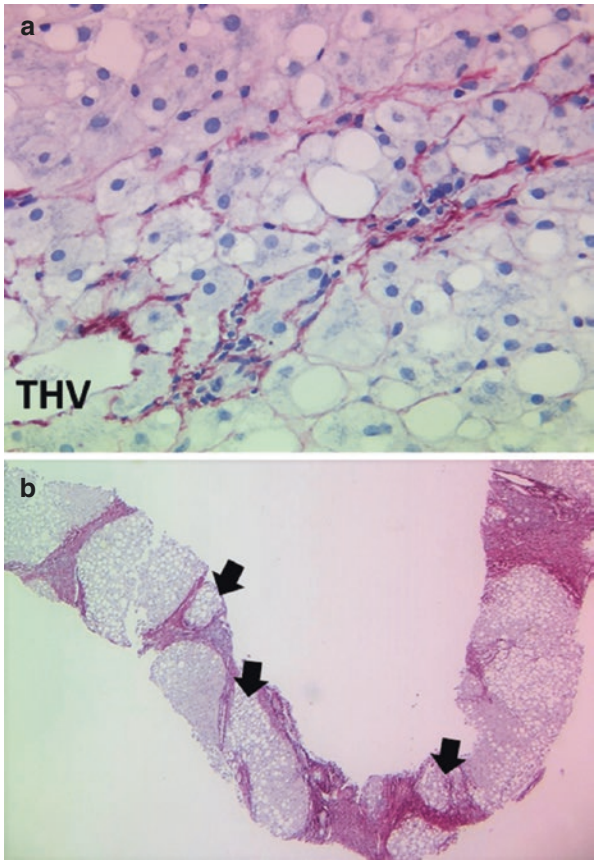

Fig. 3.4 (**a**) Nonalcoholic steatohepatitis-NASH (stage 1a NASH CRN): Thin sinusoidal and pericellular fibrosis in zone 3 close to the terminal hepatic venule (THV). Sirius red stains collagen fibers red, ×400. (**b**) Liver biopsy with NASH cirrhosis (stage 4 NASH CRN): Thick bridging fibrous septa with fully circumscribed nodules of steatotic liver parenchyma (black arrows). Sirius red stain, ×20

Ductular reaction refers to the ductular proliferation at the portal–parenchymal interface that is highlighted by keratin 7 and keratin 19 immunostains and reflects activation of hepatic progenitor cells. In NASH, ductular reaction is thought to be triggered by hepatocyte replicative arrest and is associated with portal/periportal and progressive fibrosis [25]. Isolated portal/periportal fibrosis may be seen in NASH in some bariatric cases and in children [9, 26].

Fibrosis stage has emerged as the most important prognostic factor in NAFLD predicting mortality and time to development of severe liver disease [27, 28]. Liver-related mortality in NAFLD rises dramatically with every fibrosis stage, emphasizing the necessity of accurate histological stage evaluation [29, 30]. Recently, a retrospective study based on histological data collected from two clinical trials (PIVENS and FLINT), including baseline and final biopsies, showed that fibrosis improvement is associated with resolution of NASH and improvement of semi-quantitative scores for main histological features (steatosis, ballooning, Mallory-Denk bodies, and portal inflammation) [31].

3.2.3 Other Histological Features of NAFLD/NASH

Other commonly seen nonspecific histological features in NAFLD are glycogenated nuclei in periportal (zone 1) hepatocytes seen as nuclei with central vacuolation and megamitochondria (giant mitochondria) that are round or needle-shaped eosinophilic intracytoplasmic inclusions in hepatocytes with microvesicular steatosis. Megamitochondria are thought to be the result of mitochondrial injury from lipid peroxidation or hepatocyte adaptation [4]. Mild hepatic siderosis (grade 1+, 2+) in NAFLD is common and may result from dysmetabolic iron overload. Iron granules are highlighted with special histochemical stains (Perls, Victoria Blue) within hepatocytes and/or non-parenchymal cells (macrophages, endothelial cells, etc.). The relationship of parenchymal and non-parenchymal siderosis and advanced fibrosis with HFE mutation status, iron metabolism, and insulin resistance is poorly understood and hampered by controversial evidence [32].

3.3 Histopathology of Pediatric NAFLD

NAFLD is the most common pediatric chronic liver disease in parallel with the high prevalence of obesity (approximately 30%) in this age group [33]. It is estimated that 3–11% of children and adolescents have fatty liver disease and the frequency rises to 46% in overweight and obese group [34]. NASH may be present in 25–50% of pediatric NAFLD cases and 10–25% may exhibit advanced fibrosis. Pediatric NAFLD patients can be fast progressors, eventually in need for liver transplantation [35, 36]. In two large multicenter studies including 177 and 180 children, 14% and 20% had bridging fibrosis, respectively, and only one was found with cirrhosis on biopsy [37].

Noninvasive tools for NAFLD diagnosis and fibrosis assessment currently lack validation in children, therefore, liver biopsy provides valuable information for the diagnosis, especially if interpreted by experienced hepatopathologists [38]. Differences in the histological picture from adult NAFLD and heterogeneity among pediatric cases have raised the question whether fatty liver in children represents a distinct disease entity [39]. However, it is now accepted that adolescents show a histological pattern very similar to adult fatty liver with zone 3 steatosis, lobular inflammation, ballooning, and sinusoidal fibrosis. Prepubertal, usually male children, exhibit the "zone 1 borderline pattern," characterized by steatosis mainly in periportal (zone 1) areas, accompanied by mild portal and/or lobular inflammation and portal/periportal fibrosis (Fig. 3.5). Ballooned hepatocytes, a requisite for the diagnosis of adult steatohepatitis, are uncommon and when absent a diagnosis of definite NASH cannot be made in these cases. Children with NAFLD "zone 1 borderline pattern" may progress to advanced fibrosis [37].

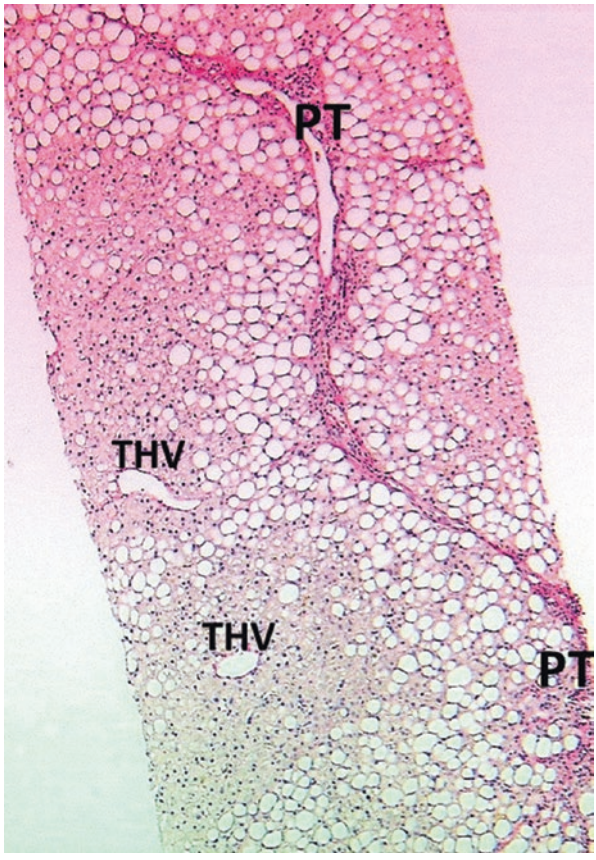

Fig. 3.5 NAFLD in a 12-year-old boy. Zone 1 borderline pattern with steatosis accentuation in periportal (zone 1) areas and portal-based bridging fibrosis. Portal tract (PT), THV (terminal hepatic venule). Sirius red stain, ×40

3.4 Differential Diagnosis of NAFLD

NAFLD shares similar histological features with many chronic liver diseases of other etiology, including ALD, drug-induced liver injury, inherited defects of metabolism, and chronic hepatitis C (CHC). The histological pattern of injury and lesion topography within the hepatic lobule (acinus) is critical for accurate morphological diagnosis, and clinical correlation is mandatory to evaluate disease etiology and reach the correct diagnosis.

3.4.1 Alcohol-Related Liver Disease

ALD and NAFLD share similar but not identical histological appearances. Distinguishing between alcoholic and nonalcoholic etiology of simple steatosis or mild steatohepatitis based on histology alone is impossible. However, in more advanced fatty liver disease, the presence and/or severity of certain histological features may sometimes point toward the correct etiology. Alcoholic steatohepatitis usually has a more severe histological picture with pronounced necroinflammatory lobular activity, numerous easily recognized MDB, which may be additionally seen in apoptotic hepatocytes and prominent satellitosis [4, 7–9, 37]. Alcoholic hepatitis may be diagnosed without steatosis. In contrast, steatosis is a required feature for the diagnosis of NASH.

Sclerosing hyaline necrosis, canalicular cholestasis, and prominent ductular reaction with cholangiolitis are more compatible with alcoholic etiology in non-cirrhotic fatty liver disease [4, 40], whereas numerous glycogenated nuclei and lipogranulomas are more commonly seen in non-cirrhotic NAFLD [4, 12]. The diffuse microvesicular steatosis that characterizes alcoholic foamy degeneration has not been described in NAFLD to date. Types of collagen may differ between fibrotic NAFLD and ALD, with type I collagen more common in the former and type III in the latter [41]. Table 3.1 summarizes the most important histological differences between non-cirrhotic ALD and NAFLD. It must be stressed that in clinical practice on an individual case basis the usefulness of these features is uncertain [4].

3.4.2 Fatty Liver Disease of Dual Alcoholic and Metabolic Etiology

People who consume excess alcohol often have metabolic risk factors, including obesity and insulin resistance, while people with NAFLD may be using modest amounts of alcohol. ALD and NAFLD pathogenetic mechanisms are common to a great extent, resulting in lipotoxicity. Moreover, ALD and NAFLD share common genetic background, mainly related to PNPLA3 and TM6SF2 gene polymorphisms [4, 42].

Table 3.1 Comparison of histological features in alcohol-related (ALD) and nonalcoholic liver disease (NAFLD) (modified from reference [4])

	ALD	NAFLD
Noncirrhotic		
Steatosis	+/−; Macrovesicular or mixed; zone 3 or panzonal, extensive microvesicular steatosis in alcoholic foamy degeneration	Required; Macrovesicular or mixed; zone 3 or panzonal in adults; panzonal or zone 1 in prepubertal children; extensive microvesicular steatosis not seen
Megamitochondria	May be prominent	+/−
Mallory-Denk bodies, zone 3	+/− Usually thick, ropy; Satellitosis common	+/−; In ballooned hepatocytes; usually thin, wispy; Satellitosis uncommon
Portal chronic inflammation	+/−; May be prominent in abstinence	+/−; May be prominent in "resolution"
Portal acute inflammation	+/− Accompanies ductular reaction; Implies cholangiolitis, pancreatitis	−
Ductular reaction	Periportal, perivenular	+/−; usually periportal
Glycogenated nuclei	+/−; less common	+/−; more common, zone 1 predilection
Iron deposition *Hepatocellular, zone 1 > 3* *Reticuloendothelial cells*	+/−; may be significant	+/−; usually mild
Fibrosis		
Zone 3 sinusoidal/ pericellular "*Chickenwire*"	+/−; Usually dense, diffuse	+/−; Usually delicate
Perivenular fibrosis	+	−
Periportal fibrosis	+/−; Ductular reaction, acute inflammation	+/−; Ductular reaction
Sclerosing hyaline necrosis *zone 3*	+	−
Veno-occlusive lesions	+	−
Cirrhosis		
Copper deposition	+/−	Uncommon
Iron deposition *Hepatocellular, reticuloendothelial cell (non-HFE patient)*	+/−	+/−
α1-Antitrypsin globules	+/−	−

In the subgroup of people with fatty liver disease of dual etiology—both alcoholic and metabolic—each one of the etiological factors may overexpose the liver to the deleterious effects of the other, potentially accelerating the progress of liver disease [43, 44]. Indeed, a recent study has shown that alcohol intake, at even low or moderate amounts, in people with the metabolic syndrome is associated with increased fibrosis [44, 45], while heavy episodic drinking has also been correlated to fibrosis progression in histologically confirmed NAFLD patients [46]. Alcohol use in NAFLD may also increase the risk of HCC [44, 47]. On the other hand, some, but not all, observational studies show beneficial effects of low or moderate alcohol consumption on cardiovascular outcomes in patients with NAFLD [42].

Both Alcoholic and metabolic Fatty Liver Disease and Both Alcoholic and metabolic SteatoHepatitis (BAFLD/BASH) are newly introduced terms used to describe cases where features of the metabolic syndrome and excessive alcohol consumption coexist [48]. From a histopathology point of view, however, in a given liver biopsy, the pathologist cannot discern the individual contribution of alcohol or metabolic factors to the development of the histological features of fatty liver disease [4].

3.5 NAFLD in Concurrence with Other Chronic Liver Disease

Given the high prevalence of fatty liver globally, it is not surprising that NAFLD and other forms of liver disease can coexist in the same patient. On the other hand, some liver diseases may be etiologically related to FLD independently from insulin resistance, such as ALD, CHC, and DILI. In these cases, it is difficult to verify if the concurrent disease is the primary culprit for steatosis or steatohepatitis or whether it aggravates already present NAFLD. In a patient with metabolic risk factors and a "bright liver" on ultrasound, when serological findings indicate autoimmune or viral etiology, current guidelines propose the use of liver biopsy to confirm or exclude NAFLD/NASH diagnosis, assess differential diagnosis, and evaluate the severity of liver injury.

In the setting of NAFLD in concurrence with other chronic liver disease, a centrilobular pattern of injury with sinusoidal/pericellular fibrosis is important for the histological diagnosis of NASH since steatosis, ballooning, and lobular inflammation are nonspecific features seen in a variety of chronic liver diseases of other etiology [4, 8, 49, 50].

3.5.1 Autoimmune Hepatitis

Non-organ-specific autoantibodies, such as anti-nuclear-antibodies (ANA), smooth muscle antibodies (SMA), and anti-mitochondrial antibodies (AMA), are more frequently detected in the serum of NAFLD patients (up to 21%) compared to the general population [51, 52]. The presence of serum autoantibodies in NAFLD has been correlated with milder steatosis while there is no association with disease activity or fibrosis in most reports [4, 8, 52].

In a recent retrospective study of 73 AIH cases, 14% and 16% had concurrent NAFL and NASH, respectively. Cirrhosis was more frequently present and mortality was higher in patients with concurrent AIH and NASH compared to those with AIH alone or AIH and NAFL [53].

3.5.2 Chronic Hepatitis C

The diagnosis of chronic hepatitis C (CHC) and concomitant fatty liver disease deserves special attention since steatosis is a common characteristic of HCV-related hepatitis. Forty percent of HCV genotype 1, 50% of genotype 2, and 86% of genotype 3 infected patients may show hepatic steatosis [54]. Metabolic parameters are mainly responsible for liver fat accumulation in HCV genotypes 1 and 2, whereas HCV genotype 3 acts directly on hepatocytes causing lipotoxic injury in a viral load depending manner [55]. Steatosis is independently correlated with liver fibrosis, possibly through the related inflammatory process [54]. Currently, there are no set criteria for diagnosing NASH concurrent with CHC. In pure CHC, steatosis is periportal or non-zonal in contrast to the centrilobular (acinar zone 3) predilection of steatosis in NASH. In cases of CHC with steatosis, the prominence of ballooned hepatocytes with MDB and the presence of centrilobular (zone 3) sinusoidal/pericellular fibrosis are features supporting concurrent NASH when there is no history of alcohol misuse [4, 9, 50, 56].

The prevalence of NASH in CHC is 4–10%, and these patients are at a higher risk for advanced fibrosis [4, 50, 56]. Patients infected with HCV genotype 3 can develop NASH even if aspects of the metabolic syndrome are not present [54, 55]. The exact effect of hepatic steatosis to treatment response with the novel direct acting antiviral agents (DAA) is not known, but it has been speculated that the lower response rate of genotype 3 to DAA could be attributed to steatosis [54].

3.5.3 Chronic Hepatitis B

Diabetes and obesity are associated with advanced fibrosis and increased risk of HCC in CHB [57]. In a large-scale meta-analysis, steatosis prevalence among chronic hepatitis B (CHB) patients was 29.6%, similar to the general population. Steatosis in CHB was related to increased body-mass index (BMI), diabetes mellitus, and other metabolic disorders, although, in contrast to CHC, no association with inflammation or fibrosis was evident [58]. Concurrent steatosis has been shown to increase the risk of HCC in the setting of CHB [59]. Accordingly, Asian-Pacific clinical guidelines for the management of hepatitis B recommend assessment of comorbidity factors such as metabolic liver disease with steatosis or steatohepatitis [60].

3.6 NAFLD in Special Populations

3.6.1 Bariatric Patients

The majority of patients undergoing bariatric surgery have fatty liver disease (66–91%) [61], while the reported prevalence of steatohepatitis is highly heterogenous (12–97%) [62]. In a recent study on 798 bariatric cases, 18% had normal hepatic histology. These were mainly females in their late thirties, usually non-Caucasian, indicating that younger female patients are eligible for bariatric surgery before liver damage becomes evident [61]. In the same study, among patients with NAFLD, 42% had definite NASH and 58% had simple fatty liver (NAFL), while only 7% had advanced fibrosis. In 5% of bariatric patients, mild inflammation, ballooning, or fibrosis was seen without steatosis, possibly indicating regressed NAFLD [49]. Liver tissue injury appeared to aggravate as trunk/limp adiposity ratio augmented, indicating that visceral rather than subcutaneous fat accumulation may be pathogenetically related to NAFLD [61].

There is no common practice regarding liver biopsy performance during bariatric surgery. Despite minimal risk of complications, the utility of liver tissue evaluation under the circumstances is unclear, leaving the decision on individual preference [62]. A liver needle biopsy is optimal for this scope because it avoids subcapsular fibrotic areas. However, usually, a wedge surgical liver biopsy is obtained. In this case, to increase accuracy of histological interpretation, the histopathologist should evaluate fibrosis at a distance of >5 mm from the capsule and exclude lesions of "surgical hepatitis" (foci of polymorphonuclear inflammation without evidence of hepatocyte necrosis/apoptosis) from the evaluation of lobular inflammation [4, 49].

Data from two large meta-analyses converge that following bariatric surgery all features of NAFLD, including steatosis, steatohepatitis, and fibrosis, are downgraded leading to reversal or significant improvement of NAFLD and NASH [63]. Indeed, biopsy-proven NASH may completely disappear after bariatric surgery in 85% of cases, in parallel with reduction in BMI and aminotransferase levels [64]. While impressive, it remains to be seen if these affects are long lasting as previous studies report significant weight gain despite the initial loss. Randomized controlled trials are necessary to increase certainty, and prospective studies should be designed to conclude on the longitudinal results of surgical intervention regarding the effect on insulin resistance and the frequency of regaining the lost weight [65].

3.6.2 Nonobese/Lean NAFLD Patients

Although the archetypal NAFLD patient is obese, it is nowadays well documented that nonobese and even lean individuals can suffer from NAFLD and NASH. Patients

with normal BMI and fatty liver disease usually have excessive visceral fat known to be associated to features of the metabolic syndrome and insulin resistance. High cholesterol and fructose diet and genetic polymorphisms have also been implicated in the pathophysiology of NAFLD in this study group [66].

Based on noninvasive diagnosis, the prevalence of nonobese NAFLD ranges from 3 to 27.5% and that of lean NAFLD from 3 to 21% [67]. In a recent study from Hong Kong on biopsy-proven NAFLD, 23.5% of the patients were nonobese and 9.4% were lean. Nonobese NAFLD patients had less severe steatosis and fibrosis [68]. A recent meta-analysis confirmed that lean NAFLD patients share more favorable histology with milder steatosis, lower necroinflammatory activity, less frequent NASH, and lower fibrosis stage compared to obese NAFLD patients [69].

3.6.3 Asians

Asians accumulate more fat in the viscera than people of other racial background after adjustment for age, gender, and BMI, and obesity-related metabolic disturbances occur at lower body weight than in Caucasians. Accordingly, cutoff values for Asian BMI have been set lower than Western BMI (overweight >23–27.5 kg/m^2 and obese ≥27.5 kg/m^2) [70]. The prevalence of NAFLD in China has been reported at 42%, which is similar to the 44% prevalence of NAFLD in Europe [71]. This similarity indicates that the prevalence and incidence of NAFLD and advanced fibrosis in Asians may be related to factors other than BMI [4, 9]. Currently, there are no data to suggest that there are differences in the histopathological features of NAFLD between Caucasian and Asian patients [4, 9]. In a recent study from Asia [68], fibrosis stage was the best prognostic factor of overall and liver-related survival in keeping with longitudinal studies on NAFLD from Europe and North America [27, 28].

3.6.4 Hispanics

Studies from the United States have shown that Hispanic-Americans are more prone to have NAFLD compared to non-Hispanic Caucasian and African-Americans [72]. Two studies of biopsy-proven NAFLD showed more severe NASH activity but lower rates of advanced fibrosis in Hispanics compared to other groups [73, 74]. Large population studies concur that NAFLD heritability is considerably higher in Hispanic-Americans (33–34%) than African-Americans (14–20%) [66]. Differences in the presence of predisposing genetic variants among ethnic groups are more likely responsible for this phenotype [75].

3.7 Grading Activity and Staging Fibrosis in NAFLD/NASH

Several semi-quantitative histopathological scoring systems have been developed specifically for NAFLD/NASH since 1999 aiming to grade necroinflammatory activity and staging fibrosis, in adults and children. These systems are mainly used to evaluate the efficacy of therapeutic interventions, to assess histological changes over time in paired biopsies in natural history studies and are useful as guidelines for the reproducible evaluation of the most significant histological features of NAFLD/NASH. In 1999, Brunt et al. proposed a method of grading NASH activity (global activity grade), based on the semi-quantitation of steatosis, lobular inflammation, and hepatocyte ballooning (Table 3.2), and a five-tiered method for staging

Table 3.2 NAFLD/NASH histological activity scoring systems

Brunt Grading System for NASH [23]			
NASH Grade	**Steatosis**	**Ballooning**	**Inflammation**
Mild *Grade 1*	1–2	Minimal	L = 1–2 P = 0–1
Moderate *Grade 2*	2–3	Present	L = 1–2 P = 1–2
Severe *Grade 3*	2–3	Marked	L = 3 P = 1–2
Steatosis: Grade 1, 0–33%; 2, 33%–66%; 3, ≥66%. *Ballooning: Zonal location noted* *Lobular inflammation-L (0–3): 0: None; 1: <2 foci/20x; 2: 2–4/20x; 3: >4/20x field* *Portal inflammation-P (0–3): 0: None; 1: Mild; 2: Moderate; 3: Marked*			
NASH Clinical Research Network Activity Score [76]			
Semi-quantitation of histological components			
Steatosis grade*	**Lobular inflammation**	**Hepatocellular ballooning**	
0: <5%	0: None	0: None	
1: 5–33%	1: <2 foci/×20 field	1: Mild, few	
2: 34–66%	2: 2–4 foci/×20 field	2: Moderate-marked, many	
3: >66%	3: >4 foci/×20 field		
NAFLD activity score (NAS): 0–8			
Steatosis (0–3) + Lobular inflammation (0–3) + Ballooning (0–2)			

SAF activity grading system for NAFLD [17]	
Semi-quantitation of histological components	
Lobular Inflammation 0–2	**Hepatocyte Ballooning 0–2**
0: None 1: ≤2 foci per 20× field 2: >2 foci per 20× field	0: None 1: Clusters of hepatocytes with round shape and pale and/or reticulated cytoplasm 2: Same as score 1 with enlarged hepatocytes (>2× normal size)
SAF ACTIVITY GRADE (A): 0–4 (steatosis score NOT included)	
Sum of scores for ballooning and lobular inflammation A_1 (A = 1): Mild activity; A_2 (A = 2): Moderate activity; A_3 & A_4 (A > 2): Severe activity * % of steatotic hepatocytes; similar grading for steatosis in the SAF scoring system	

Table 3.3 Five-tiered (stages 0–4) fibrosis staging systems for NAFLD/NASH

Brunt fibrosis staging system[a] [23]	
0	none
1	zone 3 perisinusoidal fibrosis (SF), focal or extensive
2	as above with focal or extensive periportal fibrosis
3	bridging fibrosis
4	cirrhosis, probable or definite
NASH clinical research network fibrosis staging system[a] [76]	
0	none
1	**1a** mild, zone 3 SF
	1b moderate, zone 3 SF
	1c portal/periportal fibrosis only
2	zone 3 SF AND portal/periportal fibrosis
3	bridging fibrosis
4	cirrhosis, probable or definite
SAF fibrosis staging system [17]	
FIBROSIS STAGE (F)	
F_0:	No significant fibrosis
F_1:	1a mild zone 3 SF
	1b moderate zone 3 SF
	1c portal fibrosis only
F_2:	Zone 3 SF with periportal fibrosis
F_3:	Bridging fibrosis
F_4:	Cirrhosis

[a]Fibrosis extent is assessed using Masson's trichrome stain for collagen

the characteristic fibrosis of NASH (Table 3.3) [23]. The method for the evaluation of NASH severity has been subsequently modified and is widely used today.

In 2005, the NASH Clinical Research Network (NASH CRN) sponsored by the National Institute of Diabetes and Digestive and Kidney Diseases in the United States (USA) proposed and validated a summative scoring system for NAFLD activity based on the main histological features (steatosis, lobular inflammation, and hepatocyte ballooning), which could be applied to the whole histological spectrum of NAFLD, not only NASH, and could be also used in pediatric NAFLD biopsies (Table 3.2) [76]. For the evaluation of fibrosis, NASH CRN uses a modified five-tiered staging system, based on Brunt staging, with the difference that stage 1 is further subdivided into three substages corresponding to mild (stage 1a) or moderate (stage 1b) sinusoidal fibrosis in zone 3, and portal/periportal fibrosis in the absence of zone 3 fibrosis (stage 1c). Stage 1c corresponds to a pattern of fibrosis occasionally seen in obese patients and in pediatric NAFLD/NASH (Table 3.3). The NASH CRN scoring system is approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for use in therapeutic clinical trials of NASH and has also been applied in natural history studies of NAFLD. Its value in routine practice has not been as yet evaluated.

The summative score that results from the NASH CRN NAFLD activity grading system, known as NAFLD activity score-NAS (range 0–8), does not correlate with patient prognosis (liver-related death) [77]. Although NAS has been erroneously

used in the past to classify NAFLD diagnosis in clinical studies with score <4 indicating simple NAFL and >4 suggesting NASH, it is underlined that it cannot substitute morphological diagnosis. Therefore, it is imperative that the semi-quantitative evaluation of necroinflammatory activity should be performed only after histological diagnosis is defined based on the zonal topography and pattern of injury and the global morphological assessment of the liver tissue sample. For example, a NAS = 4 may correspond to both mild steatohepatitis (moderate steatosis 2+ mild ballooning 1+ mild lobular inflammation 1) and simple NAFLD (moderate steatosis 2 + moderate lobular inflammation 2+ ballooning 0), while a NAS = 3 may correspond to both mild steatohepatitis (mild steatosis 1+ mild lobular inflammation 1+ mild ballooning 1) and simple NAFLD (moderate steatosis 2+ moderate lobular inflammation 1+ ballooning 0).

In 2012, a simple histological scoring system for NAFLF based on the semi-quantitation of steatosis-S, NAFLD activity-A, and fibrosis-F was developed by the European Fatty Liver Inhibition of Progression (FLIP) consortium (Tables 3.2 and 3.3) [17]. In contrast to the previous scoring systems, in the new one known as SAF, steatosis, although necessary for the diagnosis of NAFLD, is not included in the evaluation of disease activity, which is scored based on the sum of the scores for lobular inflammation and hepatocyte ballooning. Fibrosis is staged from 0 to 4, according to the NASH CRN, as described above. Based on the SAF score, two categories of NAFLD severity are recognized: mild NAFLD with $A < 2$ and/or $F < 2$ and severe NAFLD with $A \geq 2$ and/or $F \geq 2$. Therefore, disease severity using SAF is assessed based on hepatocyte injury, lobular inflammation and fibrosis, parameters of known prognostic significance in NAFLD, while steatosis, which is of lesser prognostic significance and may actually have a protective effect, is not taken into account [4, 17]. The SAF scoring system has not been used in phase 2B or 3 clinical trials.

The FLIP Consortium have also developed a simple diagnostic algorithm for NAFLD/NASH based on the SAF scoring system as a diagnostic aid to histopathological interpretation in an effort to reduce inter-observer variability in the diagnosis of NASH (Fig. 3.6). Using the FLIP algorithm, NASH is diagnosed only if the three cardinal features of NASH are present (steatosis, lobular inflammation, hepatocyte ballooning) [17]. In a validation study, the application of the FLIP algorithm has significantly improved the accuracy of diagnosing NAFLD and NASH of both expert hepatopathologists (from 77 to 97%) and general pathologists (from 42 to 75%) [18]. As for NAS, care should be taken to apply the FLIP algorithm after careful histological examination, as borderline cases may not be accurately classified. For example, cases with steatosis and fibrosis only or with steatosis and activity score 1 caused by mild ballooning without lobular inflammation would be diagnosed as "steatosis" using the FLIP algorithm while they could actually represent resolution of prior NASH [4]. Indeed, therapeutic intervention may result in intermediate forms of NAFLD/NASH that are difficult to classify using a dichotomous approach. The natural history of these intermediate phenotypes has not been studied in detail to date.

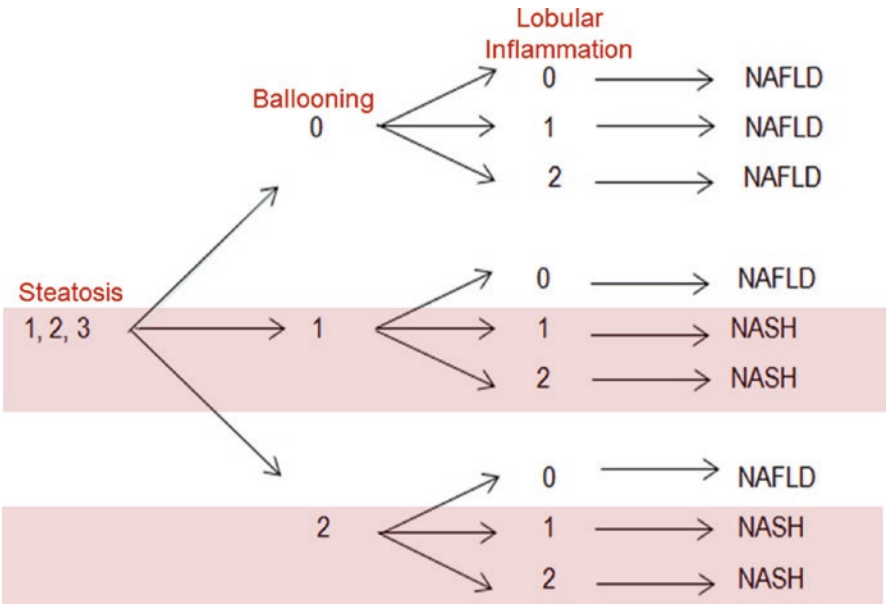

Fig. 3.6 The European Fatty Liver Inhibition of Progression (FLIP) algorithm based on the scores for steatosis (0–3), hepatocellular ballooning (0–2), and inflammation (0–2) aids stratification of NAFLD into two main diagnostic categories: NAFL versus NASH. Modified from reference [17]

3.8 The Role of Liver Biopsy in Clinical Trials

Liver biopsy is an important tool in clinical trials defining NAFLD diagnosis on baseline biopsies and assessing the extent of liver injury and the effects of the therapeutic intervention. However, there are well-known limitations to the use of liver biopsy, including sampling and inter-observer variability. These can be overcome by taking biopsies of adequate length and diameter (at least 1.5 cm in length using a 16-gauge needle), in identical fashion from the same lobe of the liver [8], using standardized scoring systems for the histological examination (described above) and/or digital image analysis (morphometry) for linear quantitative assessment of histological features, such as steatosis or fibrosis (collagen proportionate area-CPA) [8].

Currently, the accepted primary end points by the regulatory authorities in clinical trials are resolution of NASH (loss of ballooning to a score of 0, with at least a 1-point decrease in lobular inflammation) without worsening of fibrosis or reduction of fibrosis stage (without worsening of NASH), based on the NASH CRN system [77–79]. However, the results of longitudinal studies in NAFLD with more than 30 years follow-up have highlighted that histologic NASH is not an independent predictor of long-term mortality or liver-related complications [27–29]. In contrast, fibrosis stage is the most robust independent predictor of liver-related mortality in

NAFLD patients [27–29]. Therefore, fibrosis change as a primary outcome may be more adequate to assess as a clinically relevant therapeutic result although longer and/or larger trials may be required to see a measurable difference [78, 79]. In addition to the primary end points, changes in major histological features of NAFLD/NASH, including steatosis, lobular and portal inflammation, ballooning and MDB, and changes in activity scores and disease classification, should be recorded [78]. In addition to the histological end points, important secondary end points, including changes in noninvasive biomarkers, long-term outcomes, and patient-reported outcomes, also need to be taken into consideration [65].

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NAFLD and Insulin Resistance: A Multisystemic Disease

4

A. Gastaldelli

Abbreviations

AA	Amino acids
AKT	Protein kinase B
BCAA	Branched chain amino acids
DAG	Diacylglycerols
DNL	De novo lipogenesis
EGP	Endogenous glucose production
FFA	Free fatty acids
FOXO1	Forkhead box protein O1
GNG	Gluconeogenesis
Hep-IR	Hepatic insulin resistance
IHTG	Intrahepatic TG
IR	Insulin resistance
IRS-1 and IRS-2	Insulin receptor substrates
OGTT	Oral glucose tolerance test
PEPCK	Phosphoenol-pyruvate carboxykinase
Ra	Rate of appearance
T2D	Type 2 diabetes
TG	Triglyceride

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4.1 Introduction

NAFLD is an emerging metabolic disease that is affecting almost 25% of the world population [1]. In NAFLD there is a high prevalence of obesity (51%), metabolic syndrome (43%), and type 2 diabetes (T2D, 23%) [2]. Alteration in glucose and lipid metabolism and increased insulin resistance (IR) are highly common [3–7].

IR is a characteristic feature of patients with T2D and is also common in obese subjects regardless of T2D. Most of the subjects diagnosed with NAFLD are obese, so it is not surprising to find that the majority of patients with NAFLD have insulin resistance and T2D [8–10]. However, impaired insulin action is often detected also in nonobese NAFLD [3] that are as IR as obese and diabetic NAFLD [11].

IR is the inability of a known amount of endogenous (or exogenous) insulin to stimulate glucose metabolism in several organs, in particular muscle, liver, and adipose tissue (Fig. 4.1). However, insulin exerts its effects not only on glucose but also on lipids and protein metabolism. Insulin stimulates lipogenesis and protein synthesis and inhibits lipolysis and protein catabolism. In conditions of IR, the antilipolytic effect of insulin is impaired as well as its anabolic/anticatabolic effects. Thus, IR is present not only in liver and muscle but also in adipose tissue with the consequence of overflow of fatty acids to the liver that increases the risk of NAFLD [12].

In this chapter, I reviewed the current knowledge on IR in NAFLD and its impact on the metabolic cross talk among liver, muscle, and adipose tissue.

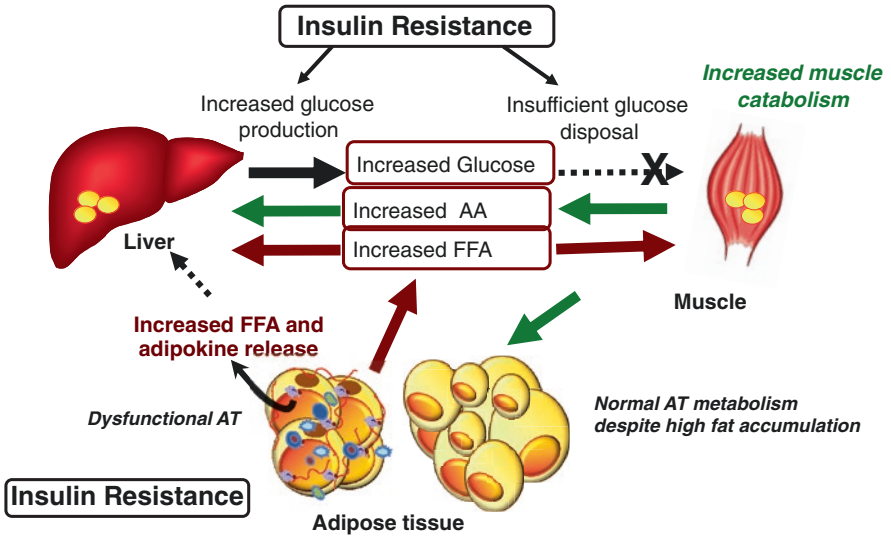

Fig. 4.1 Insulin resistance (IR) is the inability of insulin to stimulate glucose metabolism in several organs, in particular muscle, liver, and adipose tissue. This results in increased glucose production (EGP) and insufficient glucose disposal. Adipose tissue IR results in increased lipolysis and overabundance of circulating fatty acids, which in turn may contribute to the worsening of insulin resistance and ectopic fat accumulation. Insulin is exerting its effects also on protein metabolism and IR results in excess muscle catabolism and increased circulating amino acids (AA)

4.2 What Is Insulin Resistance and How We Can Measure It

Insulin is one of the most important metabolic hormones, and it is essential for the homeostasis of glucose, lipids, and protein. Insulin exerts its effects binding to its membrane receptors and is transmitted through the cell by a series of protein–protein interactions starting with the phosphorylation of insulin receptor substrates (IRS-1 and -2), which leads to the activation of PI3K and phosphorylation of Akt, which are the main signals involved in the metabolic effects of insulin [13, 14]. In the liver insulin regulates also the transcription factor Foxo1. Insulin-mediated Akt phosphorylation of Foxo1 leads to the decreased transcription of PEPCK that in turn decreases gluconeogenesis (GNG) and endogenous glucose production (EGP). Insulin also stimulates glucose uptake in muscle and liver by stimulating glycogen synthesis and glycolysis. Table 4.1 shows the most used indexes, divided in those based on samples taken during fasting state or during oral glucose tolerance test (OGTT). Hepatic and adipose tissue IR can be assessed using fasting measurements, while reliable measurements of peripheral insulin resistance are obtained using OGTT challenge.

4.2.1 Assessment of Peripheral (Muscle) Insulin Resistance

Insulin resistance in vivo is assessed in several ways. The gold standard method is the euglycemic hyperinsulinemic clamp where insulin is infused in pharmacological doses and glucose is infused along to maintain plasma glucose concentration at constant levels (around 5 mmol/L) [15]. An insulin infusion rate of 40 mU/min/m^2 or higher is infused to evaluate peripheral insulin resistance since at this dose endogenous glucose production (EGP) is almost suppressed. Given the complexity of the clamp, several indexes have been developed and used to assess the degree of insulin resistance [16] and summarized in Table 4.1.

Fasting indexes are HOMA-IR and QUICKI that are based on the product of fasting glucose and insulin concentrations. They are widely used since they are measured after an overnight fasting. Recently, Isokuortti et al. have determined the HOMA-IR cutoff for NAFLD (liver fat ≥5.56%, based on the Dallas Heart Study) that was 2.0 [17]. However, this cutoff should be taken cautiously since the same authors found a large inter-laboratory variation for HOMA-IR (25%) due mainly to inter-assay variation in insulin (25%) rather than glucose (5%) measurements [17]. The most reliable indexes are based on glucose and insulin concentrations measured during the OGTT. The most used are the Matsuda index [18] and the OGIS index [19]. The last one has the advantage that is an estimate of glucose clearance based on a mathematical model. Not only OGIS correlates with both glucose disposal during the clamp but also with glucose clearance during OGTT [16]. OGIS has been used in a few studies in subjects with NAFLD and was found also associated to increased liver fibrosis [16, 20]. It should be considered that in subjects with diabetes insulin secretion is often impaired [21] and thus glucose concentrations (and consequently HOMA and OGTT indexes) are

Table 4.1 Formulas for surrogate indexes of insulin resistance/insulin sensitivity using fasting or OGTT measurements of metabolic parameters

Based on	Tissue	Formula
Fasting measurements		
HOMA-IR	Peripheral/liver	(I_0 mU/mL × G_0 mmol/L)/22.5
QUICKI	Peripheral/liver	1/(log I_0 mU/mL + log G_0 mg/dL)
FIRI	Peripheral	(I_0 mU/mL × G_0 mg/dL)/25
IGR	Peripheral	I_0 mU/mL × G_0 mg/dL
ISI Bennett	Peripheral	1/(ln G_0 mg/dL × ln I_0 mU/L)
TG/HDL-Chol	Liver	Tg/HDL-Chol
Hep-IR	Liver	EGP × I_0 mU/L
Adipo-IR	Adipose tissue	FFA × I_0 mU/L
Lipo-IR	Adipose tissue	RaGly × I_0 mU/L
OGTT measurements		
OGIS	Peripheral	f (G_0, G_{90}, G_{120}, I_0, I_{90}, D)[a]
ISI Matsuda	Peripheral	$10^4/\sqrt{}$ [(G_0 mg/dL × I_0 mU/mL) × (G_{mean} × I_{mean})]
SiOGTT	Peripheral	1/[log(G_0 + G_{30} + G_{90} + G_{120}) mg/dL + log (I_0 + I_{30} + I_{90} + I_{120}) mU/mL]
ISI Stumvoll	Peripheral	0.157 − 0.00004576 × I_{120}(pmol/L) − 0.000299 × I_0(pmol/L) − 0.00519 × G_{120}(mmol/L)
BIGTT	Peripheral	exp (4.9 − (0.00402 × I_0 pmol/L) − (0.000556 × I_{30} pmol/L) − (0.00127 × I_{90} pmol/L) − (0.152 × G_0 mmol/L) − (0.00871 × G_{30} mmol/L) − (0.0373 × G_{120} mmol/L) − (0.145 × gender) − (0.0376 × BMI)
$eMCR^{dem}$	Peripheral	18.8 − 0.271 × BMI − 0.0052 × I_{120} pmol/L − 0.27 × G_{90} mmol/L
$eMCR^{nodem}$	Peripheral	13 − 0.0042 × I_{120} pmol/L − 0.384 × G_{90} mmol/L − 0.0209 × I_0 pmol/L
HepIR OGTT	Liver	(G_0 mg/dL + G_{30} mg/dL)/100/2 × (I_0 mU/mL + I_{30} mU/mL)/2
LIRI	Liver	−0.091 + log (I mean × 6) × 0.4 + log (FM/weight × 100) × 0.346 − log HDL-C mg/dL × 0.408 + log BMI × 0.435

Note. *ALB* albumin, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *AUC* area under the receiver operating curve, *BIGTT* β-cell function, insulin sensitivity index derived from oral glucose tolerance test, *BMI* body mass index, $eMCR^{dem}$ metabolic clearance rate estimation including demographic parameters, $eMCR^{nodem}$ metabolic clearance rate estimation without demographic parameters, *FIRI* fasting insulin resistance index, *HDL-C* high density lipoprotein cholesterol, *G* glucose, *HepIR OGTT* hepatic insulin resistance index, *HOMA* homeostasis model of assessment, *I* insulin, *IFG* impaired fasting glucose, *IGR* insulin to glucose ratio, *IR* insulin resistance, *ISI* insulin sensitivity index, *LIRI* liver insulin resistance index, *NFS* non-alcoholic fatty liver disease fibrosis score, *OGIS* oral glucose insulin sensitivity index, *QUICKI* quantitative insulin sensitivity check index, *SiOGTT* insulin sensitivity index derived from oral glucose tolerance test, *TG* triglycerides

[a]G_0, G_{90} and G_{120} are the plasma concentration of glucose measured at baseline, 90 and 120 min during OGTT; I_0, and I_{90} are the plasma concentration of insulin measured at baseline and 90 min during OGTT. D is the oral glucose dose (g/m^2 body surface area). The formula can be found at the following website: http://webmet.pd.cnr.it/ogis/

altered not because of insulin resistance but because of impaired insulin secretion [22]. For this reason, in diabetic subjects only the hyperinsulinemic euglycemic clamp or the infusion of tracers can give a reliable measurement of muscle insulin resistance.

4.2.2 Assessment of Endogenous Glucose Production and Hepatic Insulin Resistance

In fasting state glucose is produced mainly by the liver (90%) and in part by the kidney (max 10%) [23]. Endogenous glucose production (EGP) can be estimated noninvasively by the infusion of a tracer (i.e., glucose labelled with either a radioactive or a stable isotope). The euglycemic hyperinsulinemic clamp with lower doses of insulin (e.g., 10 mU/min/m^2) is used together with the infusion of tracers to measure hepatic insulin resistance (given by the changes in EGP). A measure of hepatic insulin resistance (Hep-IR) is the % suppression during insulin infusion [24]. The dose response insulin-EGP is hyperbolic (Fig. 4.2) and thus the product of insulin times EGP is a surrogate measure of hepatic IR [24, 25]. For this reason, Hep-IR is often and more easily estimated in fasting state [16, 25]. Other indexes have been

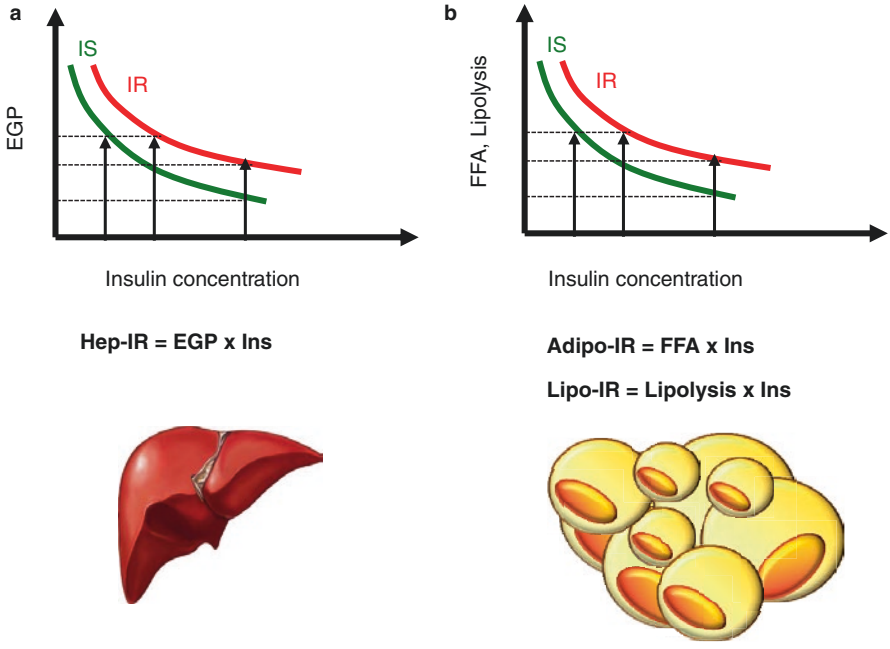

Fig. 4.2 The hyperbolic function relates insulin concentration to glucose production (EGP) (Panel **a**) or to FFA/Lipolysis (Panel **b**). As subjects become more insulin-resistant, the curve moves to the right, meaning that higher insulin concentrations are needed to maintain the same rates of lipolysis, EGP, or FFA concentrations. These relationships are true in both fasting and hyperinsulinemic state (redrawn from [24])

derived using OGTT data without tracer infusion and validated against the tracers [26, 27] (Table 4.1). However, these indexes were never tested in large cohort of subjects or after intervention (e.g., weight loss or drug).

4.2.3 Assessment of Lipolysis and Adipose Tissue Insulin Resistance

Lipolysis, i.e., the rate of adipose tissue triglyceride (TG) hydrolysis, is measured by the infusion of labeled glycerol and calculating rate of appearance (Ra-glycerol) since the free fatty acids (FFA) can be retained and re-esterified to TG [10]. Thus FFA release reflects on in part lipolysis. On the other hand, glycerol cannot be used for TG synthesis since the adipocytes lack the enzyme glycerol kinase [10]. The hydrolysis of one mole of TG results in the release of a mole of glycerol into the systemic circulation.

The euglycemic hyperinsulinemic clamp with lower doses of insulin (e.g., 10 mU/min/m^2) is used to measure adipose tissue IR measuring the suppression of free fatty acids or lipolysis. Similar to EGP also the dose response insulin-FFA concentrations or insulin-lipolysis follow a hyperbolic curve (Fig. 4.2) [3, 24, 28, 29]. Thus, the product of insulin times FFA (Adipo-IR) or the product of insulin times Ra-glycerol (Lipo-IR) are surrogates measure of adipose tissue IR in fasting state [28]. As stated above since in subjects with diabetes insulin secretion is often impaired (especially in postprandial state) only fasting Adipo-IR is reliable while OGTT suppression of FFA does not follow a hyperbolic relationship [3, 28].

4.3 Insulin Resistance: Impact of the Liver-Pancreas Cross Talk

The pancreas has an important role in the regulation of glucose homeostasis through the secretion of vital hormones, like insulin and glucagon. The pancreas releases the hormones directly into the portal vein, and thus they first reach the liver since their primary role is the regulation of glucose production and the maintenance of glucose concentration (Fig. 4.3). In the following paragraphs, I will discuss the mechanism of insulin and glucagon secretion and the importance of the cross-talk liver-pancreas I NAFLD.

4.3.1 Insulin Secretion and Clearance in NAFLD

Insulin is secreted by the pancreatic β-cells in response to hyperglycemia and is important to maintain the glucose concentrations within a tight range [12]. The beta cells produce proinsulin, and the enzymatic cleavage of proinsulin results in equimolar secretion of insulin and c-peptide into the portal vein [12]. Most of secreted

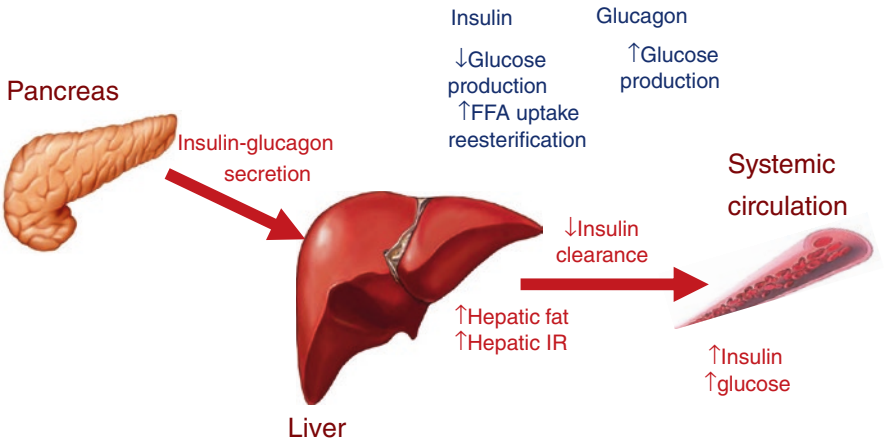

Fig. 4.3 The pancreas main hormones are insulin and glucagon that are secreted into the portal vein and thus they first reach the liver and then the systemic circulation. The main actions of insulin in the liver are the inhibition of EGP and the increased lipogenesis. Glucagon increases EGP thus contributing to hepatic IR. Great part of the insulin secreted is degraded in the liver during the first pass. In NAFLD insulin clearance is decreased in proportion to hepatic fat

insulin is degraded by the liver and in part by the kidney and the muscle (around 60%); on the other hand, c-peptide is not degraded by the liver, but it is mainly excreted through the kidney [30], and for this reason it is used to estimate prehepatic insulin secretion and insulin clearance [31, 32].

The main action of insulin is to suppress EGP, to promote glycogen synthesis and store glucose in the liver and in the muscle, to increase glycolysis (Figs. 4.3 and 4.4). Moreover, the effect on the adipose tissue is to inhibit lipolysis and promote lipogenesis also by stimulating glucose uptake in the adipose tissue where it is converted to glycerol-3P and used to synthesize TG.

Increased peripheral insulin concentration is a compensatory mechanism to overcome peripheral insulin resistance since more insulin is required to have the same metabolic effects [12, 33]. The liver metabolizes most of the secreted insulin and by reducing insulin clearance acts as a modulator of peripheral insulin concentrations following the increased insulin demand due to peripheral insulin resistance. In the pathophysiology of type 2 diabetes, insulin secretion is increased following as subjects progress from NGT to IGT, but when beta cell failure causes a decrease in insulin secretion, they develop type 2 diabetes [32, 33].

In insulin resistance state insulin clearance is decreased, contributing to peripheral hyperinsulinemia [31] (Fig. 4.3). Several studies have demonstrated that subjects with NAFLD have reduced insulin clearance proportionally to the degree of liver fat [4, 25, 34, 35]. However, the mechanisms that regulate hepatic insulin clearance are still unknown. Moreover, insulin clearance is not a static process but is rather influenced by several factors, like nutrient intake and some hormones.

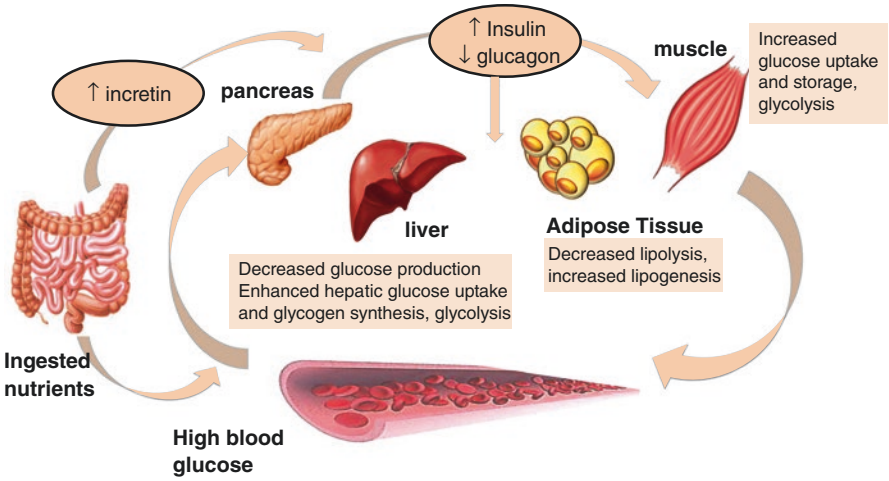

Fig. 4.4 In response to increased blood glucose, e.g., after a meal or OGTT, insulin is increased and glucagon decreased. Ingested glucose stimulates the release of incretin hormones like GLP-1 and GIP by the intestine, which stimulate insulin secretion and inhibit glucagon release. The figure shows main action of insulin and glucagon on liver, muscle, and adipose tissue. In IR state most of insulin actions are impaired in all tissues

4.3.2 Glucagon Secretion in NAFLD

Glucagon is the other important hormone secreted by the α-cells of the pancreas, with opposite actions compared to insulin [12] (Figs. 4.3 and 4.4). The α-cells produce pro-glucagon, a 160-amino-acid polypeptide, and by enzymatic cleavage, glucagon is secreted into the portal vein [12]. Proglucagon is produced also by the intestinal L-cell [12, 36, 37] although different enzymes in a tissue-specific manner are converting proglucagon to glucagon, GLP-1, and other peptides like GLP-2 or oxyntomodulin [12].

Glucagon regulates hepatic metabolism by stimulating gluconeogenesis, glycogenolysis, and net hepatic glucose output [38–40] (Figs. 4.3 and 4.4). Thus, insulin and glucagon have opposite effects on glucose metabolism. In T2D fasting plasma glucagon levels are increased despite the hyperglycemia and fail to be reduced by the postprandial hyperinsulinemia observed after meal ingestion [33, 40–43]. In NAFLD, glucagon concentrations are increased [44], even in nondiabetic subjects, possibly contributing to increased EGP and hepatic insulin resistance.

4.3.3 Incretin Effect on Insulin Secretion

The incretins (glucagon-like peptide 1, GLP-1, and glucose-dependent insulinotropic polypeptide, GIP) are hormones that are secreted by the intestinal cells in response to nutrients (Fig. 4.4) and are able to potentiate the insulin secretion [45].

GLP-1 is produced from enzymatic cleavage of proglucagon produced by the intestinal epithelial L cells. However, since proglucagon is produced also by pancreatic α-cells, both GLP-1 and glucagon can be released by the pancreas [46] and the gastrointestinal tract [37].

GLP-1 and glucagon are tightly related since GLP-1 not only stimulates insulin secretion but also inhibits glucagon release (Fig. 4.3). GLP-1 modulates hepatic, but not peripheral glucose metabolism, by suppressing EGP independently of glucagon [12, 47–51]. A new study just showed that insulin can regulates the α-cells and promotes the release of GLP-1 in a time- and dose-dependent manner under high-glucose conditions [52]. Thus, GLP-1 is important not only because it stimulates insulin secretion but also for its independent effect on hepatic insulin resistance. This is very important since GLP-1 receptor agonists (GLP-1RA) are a new class of antidiabetic drugs that are important in reducing hepatic fat and improving hepatic insulin sensitivity by decreasing EGP and increasing hepatic glucose uptake [47, 53–56].

Only few studies have looked at GLP-1 action in NAFLD, finding that it is often impaired although not always its secretion is compromised [44, 57]. Treatment of NAFLD with GLP-1RA has been shown to be effective [53, 54, 56] although the mechanisms of action are still not completely elucidated. The effect on adipose tissue and lipolysis is controversial although the treatment with GLP-1RA is effective on weight loss [58], and improves adipo-IR and lipotoxicity [47, 54].

4.4 Muscle Insulin Resistance in NAFLD

4.4.1 Impact on Glucose Metabolism

Impaired muscle glucose uptake and disposal are the principal defects associated with peripheral insulin-resistant state (Fig. 4.1). Most of the subjects with NAFLD have reduced muscle insulin sensitivity independent of obesity or diabetes [3, 7, 16, 25, 59, 60]. Moreover, muscle IR is present long before significant TG accumulation in the liver (at 1.5%) as shown by the recent paper by Bril et al. [59].

In NAFLD, decreased insulin-stimulated glucose disposal during the hyperinsulinemic clamp is proportional to hepatic fat accumulation and mainly due to a significant reduction in non-oxidative glucose disposal comparable with the one observed in T2D without NAFLD [11, 25, 59]. Also when measured by indexes derived by OGTT, insulin resistance was higher in subjects with NAFLD, and this is evident already in children [61].

In nondiabetic subjects with NAFLD, glucose tolerance seems to be independent of the degree of hepatic steatosis, while nondiabetic NAFLD with significant fibrosis (F2–F4) has worse glucose tolerance independent of obesity [16] (Fig. 4.5). This is confirmed by the strong inverse association between insulin sensitivity measured by OGIS and degree of liver fibrosis [16, 20].

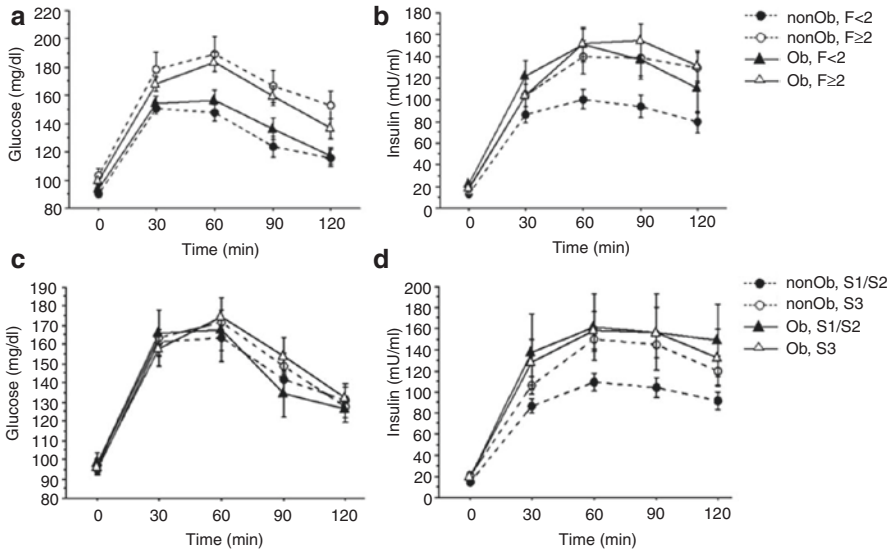

Fig. 4.5 Glucose and insulin curves during OGTT in nondiabetic subjects with NAFLD according to the presence of obesity and the stage of fibrosis (**a**, **b**) or the degree of steatosis (**c**, **d**) (n = 145) (from [16] with permission)

4.4.2 Impact on Protein Metabolism

The muscle is where protein are stored. Insulin regulates also protein metabolism by stimulating protein synthesis and reducing protein catabolism. In insulin-resistant state, despite high insulin concentrations, protein catabolism is not suppressed (Fig. 4.1) and fasting amino acid concentrations, in particular the concentrations of essential amino acids, like branched chain amino acids (BCAA), are increased [62–64]. Several studies have reported increased fasting BCAA concentrations in NAFLD also related to the severity of this disease, in particular to presence of NASH and fibrosis [62, 65–67]. BCAA have been associated also to hepatic IR since they stimulate mTOR1 [64]. However, if BCAA or other amino acids are simple biomarkers of IR or active players has still to be demonstrated. What is known is that subjects with NAFLD have decreased lean body mass and are more sarcopenic compared to subjects without NAFLD [68–70]; this condition is worsened in subjects with fibrosis F3–F4 [70]. We have hypothesized that this might be associated to muscle IR, i.e., reduced protein net balance due to increased protein catabolism and reduced protein anabolism [62].

4.5 Hepatic Insulin Resistance in NAFLD

4.5.1 Impact on Hepatic Glucose Production

The liver is the principal organ that produces glucose (EGP) [23]. Hepatic insulin resistance is defined as a defect of insulin to suppress EGP during fasting and/or

during insulin infusion (Fig. 4.1). Hepatic IR is strongly associated to hepatic fat accumulation [25, 59]. In general fasting hepatic IR is increased proportionally to the degree of hepatic steatosis and is already present even when hepatic TG are less than 5% [25, 59]. It is important to note that nondiabetic subjects with NAFLD have increased fasting hepatic insulin resistance compared to non-NAFLD and similar to T2D without NAFLD and having T2D and NAFLD further increases hepatic IR [11, 25, 59]. Ortiz-Lopez et al. reported that in NAFLD with normal glucose tolerance (NGT), insulin-mediated suppression of EGP is preserved [71]. However, it has been shown that even the % suppression is similar, the dose response insulin-EGP is shifted to the right, indicating the need of higher insulin concentrations than subjects without NAFLD to suppress EGP or in other words they are more insulin-resistant [3]. In general, having have prediabetes or T2D is in general associated to lower suppression of EGP [25, 71].

4.5.2 Impact on Gluconeogenesis

The liver produces glucose through glycogenolysis and gluconeogenesis (GNG). After overnight fasting, more than 50% of the glucose is synthesized from gluconeogenic precursors such as lactate/pyruvate, glucogenic amino acids, and glycerol [38, 72, 73]. Glycerol used for gluconeogenesis comes mainly from the hydrolysis of triglycerides in the adipose tissue, while the amino acids comes from muscular proteolysis (Fig. 4.1). Almost all amino acids are glucogenic and they are alanine, glutamic acid and glutamine, glycine, arginine, asparagine, aspartic acid, cysteine, serine, valine, phenylalanine, tyrosine, isoleucine, tryptophan, methionine, histidine, threonine, proline, while lysine and leucine are used to produce ketone bodies. Fasting endogenous glucose production is tightly regulated and in nondiabetic subjects, endogenous glucose production (EGP) by the liver is relatively similar among subjects when whole body fluxes are normalized by lean body mass [38, 74]. In diabetic subjects, EGP is increased proportionally to fasting plasma glucose [23, 33, 38].

High rates of glucose production are mainly due to increased GNG flux [38]. In NAFLD, GNG fluxes tend to be increased as a consequence of increased glycerol and amino acid concentrations [75], indicating increased peripheral lipolysis and protein catabolism. The visceral fat is often increased in these subjects, and it is related to increased insulin resistance [4]. This tissue is also highly lipolytic making VF an important contributor of glucogenic substrates since it is drained by the portal vein. We have shown that GNG flux is increased proportionally to VF, while there is no correlation between GNG and the amount of TG stored in the liver [4, 25, 76].

Hepatic IR is also due to impaired suppression of GNG since it has been shown that glycogenolytic fluxes are similarly suppressed in non-diabetic and T2D subjects during the euglycemic hyperinsulinemic clamp [77]. Insulin exerts its effects on the liver by reducing glycogenolysis and after a meal by stimulating glycogen synthesis (Fig. 4.4). The effects on the gluconeogenesis are mild and indirect since the release of most of gluconeogenic precursors (i.e., glycerol and amino acids) is insulin-dependent (Fig. 4.1) and high insulin concentrations decrease lipolysis (i.e.,

glycerol release from the adipose tissue) and proteolysis (i.e., amino acid release from the muscle) both in fasting and feeding state [14]. So it is likely that increased gluconeogenesis is a compensatory mechanism for the hepatic metabolism of substrates in excess [10, 75].

4.5.3 Impact on Hepatic De Novo Lipogenesis

Insulin also promotes lipogenesis and triglyceride production in the liver. Moreover, insulin promotes the de novo synthesis of fatty acid (DNL) first palmitate and then for elongation stearic acid, and for desaturation palmitoleic acid and oleic acid. The main DNL precursors are carbohydrates that, if they cannot be oxidized or stored as glycogen, are then stored as TG [10, 75, 78, 79]. DNL is increased in subjects with NAFLD, particularly after high carbohydrate and/or high fructose intake [10, 78, 80]. Donnelly et al. have estimated that in NAFLD about 26% of intrahepatic TG (IHTG) are from DNL, 59% from FFA (i.e., derived from peripheral lipolysis), and 15% from TG of the diet [80]. In subjects with NAFLD, DNL is increased up to three times the rate observed in healthy subjects [81]. However, not all subjects with NAFLD have increased DNL, particularly if they have *PNPLA3* 148M allele since these subjects have lower DNL and expression of the lipogenic transcription factor SREBP1c [82]. Moreover, DNL rates are highly dependent on meal composition. We have recently shown that carbohydrate overfeeding stimulated DNL by +98% and increased IHTG +33% [79]; also fat overfeeding increased IHTG, +55% if the diet was rich in saturated fat vs +15% for diet rich in unsaturated fat +15% ($p < 0.05$), but this was due to excess fat since the rates of hepatic DNL were unchanged compared to baseline [79].

4.6 Adipose Tissue Insulin Resistance in NAFLD

4.6.1 Impact on Lipolysis

IR is present not only in the liver and the muscle but also at the level of the adipose tissue (Fig. 4.1). The main effect of insulin in the adipose tissue is glucose uptake for triglyceride synthesis and inhibition of lipolysis. In presence of IR, there is an excess lipolysis and FFA release despite high circulating levels of insulin. This is more evident during fasting state when insulin is low [11, 28, 59, 83–85]. However, also diet composition seems to be implicated in the worsening of adipose tissue IR. We have shown that overfeeding with saturated fatty acids increased fasting lipolysis compared to diet with similar caloric intake but rich in unsaturated fat or carbohydrates [79]. Excess FFA from the adipose tissue determines an overflow to the liver and other organs (Fig. 4.1) which in presence of high insulin concentrations favors intracellular TG re-esterification and ectopic fat accumulation not only in the liver but also in other organs including pancreas and heart [21, 86].

4.6.2 Impact on Lipogenesis

Insulin also promotes lipogenesis and adiposity (Fig. 4.4). Excess carbohydrate promotes adipogenesis since glucose is also used as a precursor of glycerol-3P and used for TG synthesis. DNL occurs mainly in the liver although we cannot exclude that it might be active also in the adipose tissue [87]. Ectopic fat accumulates only when the subcutaneous adipose tissue is not able to store excess fat and glucose since lipogenesis is impaired [86, 88, 89]. This adipose tissue is often found inflamed, resistant to the antilipolytic effect of insulin, with increased release of pro-inflammatory adipokines and reduced secretion of adiponectin [86, 88, 89]. This not only impairs fatty acid oxidation but also promotes the synthesis of lipotoxic lipids that may act as signals that worsen IR, glucose, and lipid metabolism (see below).

4.7 Lipotoxicity, Glucotoxicity, and IR

Lipotoxicity is the accumulation of lipids that impair metabolic signaling, leading to alteration in glucose and lipid metabolism, insulin resistance, and impaired insulin secretion [90]. Impaired triglyceride synthesis or partial hydrolysis of TG can lead to the production and accumulation of lipid species like diacylglycerols (DAG) and ceramides [5, 14, 91, 92]. Production of lipotoxic metabolites like DAG can cause insulin resistance by activating PKCε [13, 93]. In humans, lipid infusion induces muscle IR by transient increase in total and cytosolic DAG content [93]. The activated PKCε binds to the insulin receptor and inhibits its tyrosine kinase activity interfering with the ability of insulin to phosphorylate IRS-2 on tyrosine residues. Hepatic cytosolic DAG were observed also in human livers of subjects with NAFLD and correlated with activation of PKCε [94]. A stepwise increase in DAG and the product/precursor ratio (TAG/DAG) was observed from normal livers to NAFL to NASH [92].

Other lipotoxic compounds are ceramides and in general saturated fat. However, total hepatic ceramides are often similar among NAFLD/NASH and controls [92, 94]. This is likely because increased hepatic ceramide accumulation and/or de novo synthesis is more associated to presence of insulin resistance rather than NAFLD due to genetic predisposition [91, 95].

Lipotoxicity has been almost exclusively attributed to saturated fat that either comes from the diet or is synthesized from de novo lipogenesis (DNL). Studies in cells have shown that the incubation with oleic acid (18:1) results in immediate incorporation into triglyceride (TG) and increases TG accumulation. On the other hand, the incubation with palmitic acid (C16:0) results in poor incorporation into triglyceride and causes apoptosis [96]. The co-incubation of C18:1 and C16:0 reduces apoptosis and stimulates palmitate incorporation into TG [96]. However, when triglyceride synthesis is impaired, e.g., in cells from *Dgat1* null mice, both incubation with oleate and palmitate leads to lipotoxicity [96], indicating that accumulation of excess FA in cellular triglyceride stores may be protective against lipotoxicity.

Glucotoxicity, i.e., the toxic effects of hyperglycemia and excess carbohydrate intake on cells and tissues, is as harmful as lipotoxicity [5]. As previously discussed, hyperglycemia and excess carbohydrate intake can favor DNL, i.e., synthesis of palmitate (a saturated fat and a precursor of ceramides and other lipotoxic lipids) [5, 79]. Glucotoxicity and lipotoxicity are closely interrelated, and both contribute to the deterioration of insulin resistance and impaired insulin secretion [5, 90]. In particular, glucotoxicity alters IRS-1 signal, promotes JNK activation, and determines IR not only in liver but also in muscle, initiating a vicious cycle [5].

4.8 Genetic Vs Metabolic NAFLD

Although NAFLD is not a genetic disease, several polymorphisms have been associated to increased risk of development and progression of NAFLD showing that subjects carrying the gene variant for PNPLA3, hypo-betalipoproteinemia, DGAT, or TM6SF2 are more likely to have NAFLD [97–99]. An interesting observation was that although these subjects have NAFLD, their insulin-resistant state is no different from subjects without the gene variant and no NAFLD [4, 98]. Moreover, when subjects with NAFLD homozygous either for the rs738409 PNPLA3 G allele (PNPLA3-148MM) or the C allele (PNPLA3-148II) were placed on a hypocaloric low-carbohydrate diet for 6 days, those at high risk of NAFLD (with the G allele) had a better metabolic outcome with higher decrease in steatosis and better improvement in peripheral IR despite similar weight loss [100]. The PNPLA3 protein has lipase activity towards TG in hepatocytes and retinyl esters in hepatic stellate cells; the I148M substitution leads to a loss of function promoting intrahepatic TG accumulation [101]. PNPLA3 variant was not associated to alteration in peripheral lipolysis or hepatic fatty acid oxidation when subjects with NAFLD were matched for hepatic triglyceride accumulation [100]. On the other hand in TM6SF2 E167K variant carriers hepatic lipid synthesis from unsaturated fatty acids is impaired [79] and together with reduced VLDL secretion could contribute to increased intrahepatic TG [102].

However, it should be noted that different mechanisms explain the pathophysiology of metabolic NAFLD vs genetic NAFLD. In metabolic NAFLD, the subcutaneous adipose tissue is not able to store excess caloric intake, then fat accumulates as ectopic fat in other tissues like liver, muscle, pancreas, and heart [86, 88, 89]. This has also been supported by genetic studies [103, 104]; using integrative genomic approaches these authors have found that a cluster of genes associated with insulin resistance (of which the most important is *PPARG*) was also associated to a reduced capacity of subcutaneous tissue to expand, resulting in ectopic fat accumulation, NAFLD, and higher visceral-to-subcutaneous adipose tissue ratio [103, 104].

4.9 Insulin Resistance and NAFLD: Chicken or Egg?

Although it is recognized that IR is strongly associated to NAFLD, if IR precedes/causes NAFLD [105] or the other way around [106] has been long debated. Recent cross-sectional studies have shown that impairment in peripheral insulin sensitivity is present already in subjects with minimal hepatic TG accumulation (i.e., less than 5%) [59]. Currently there are no longitudinal studies that have properly addressed this point. This is due to many reasons, mainly because (1) data are lacking, as NAFLD has been recognized as a metabolic disease only in recent years and (2) the assessment of presence of IR is no trivial (see previous paragraphs). Metabolic studies in subjects carrying genetic risk factors for NAFLD and overfeeding studies involving non-IR subjects helped answering, at list in part, this question. It is now recognized that NAFLD has two main phenotypes: genetic (type 1) vs metabolic (type 2) NAFLD but only metabolic NAFLD is associated to IR [4, 7, 107].

Overfeeding/inactivity studies of non-IR subjects helped understanding the mechanism of development of NAFLD/IR. Several overfeeding studies have shown that the decrease in insulin sensitivity precedes the development of NAFLD. Knudesen et al. showed that 14 days of inactivity and overfeeding (+50%) induced IR as early as day 3, while body fat and visceral fat were increased significantly only after 14 days [108]. The recent paper by Peterson et al. has shown that overfeeding by 40% for 8 weeks (56 days) decreased peripheral glucose disposal, in particular non-oxidative disposal rate, at low (10 mU/min·m^2) but not at high (50 mU/min·m^2) insulin infusion rates and although it increased body weight by 7.6 kg (of which +4.2 kg of body fat), there was no clinically significant change in hepatic fat that was 1.5% at baseline and 2.2% at the end of study [109]. However, also visceral fat was low at baseline (0.58 kg) and 0.94 kg at the end of study.

Although large prospective studies on this topic are still lacking, it seems that only subjects with the "metabolic" NAFLD are more insulin-resistant and at increased risk of T2D [9–11], while subjects with the "genetic" NAFLD are more at risk of HCC and chronic liver disease [7, 99].

4.10 Conclusions

In subjects with NAFLD, IR is more pronounced at the level of the muscle, where glucose uptake is reduced, but it is present also in the liver, where insulin does not properly suppress hepatic glucose production, and in the adipose tissue, where peripheral lipolysis is high despite high insulin concentrations [8, 25, 59]. Genetic NAFLD dissociates from metabolic NAFLD since very often these subjects do not have IR or increased DNL.

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Etiopathogenesis of NAFLD: Diet, Gut, and NASH

5

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5.1 Introduction

The pathogenesis of nonalcoholic fatty liver disease (NAFLD) is based upon a "multiple hit theory" that consists in the synergic and/or consequential role of genetics and environmental factors (sedentary lifestyle and dietary habits) in liver fat accumulation, inflammation, and fibrogenesis.

The pathophysiology of NAFLD involves several factors that play a key role in the genesis and progression of the disease. Among the main actors, it is possible to identify genetic, metabolic, and environmental factors. Physical activity, energy consumption, and caloric intake influence the regulation of insulin metabolism, contributing to the development of insulin resistance and consequent accumulation of fat within the liver [1].

Raising evidence indicates that gut microbiota is deeply implicated in NAFLD pathogenesis and progression to NASH and liver fibrosis. Gut microbiota composition and gene expression (microbiome) are independently linked to NAFLD and also to many of other known risk factors such as obesity, insulin resistance, and intestinal permeability. Moreover, it is strictly regulated by dietary intake and lifestyle; this leads to a complex and not fully understood pathogenic scenario.

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In recent years, the advanced knowledge of gut microbiota and its relationship with human body allowed to identify different mechanisms of interaction between enteric flora and the development of metabolic diseases. Following these new concepts, numerous studies, in both murine models and humans, have been carried out with the aim of analyzing the relationship between gut microbiome and metabolic diseases [2].

5.2 Gut–Liver Axis and NAFLD/NASH

5.2.1 Gut Dysbiosis

The involvement of the gut microbiota in gut–liver axis alteration is complex and multifactorial.

Intestinal microbiota is a critical factor in the pathogenesis of NAFLD. Germ-free animal models are resistant to diet-induced obesity, liver steatosis, and insulin resistance, and colonization of germ-free mice with a "normal" gut microbiota harvested from the cecum of "normal" mice produced a 60% increase in body fat content, insulin resistance, and a twofold increase in hepatic triglyceride content [3]. It is unclear if this effect can be entirely explained by a greater capacity to extract calories from food.

In mice, gut microbiota transplantation associated with a high-fat diet replicated NAFLD phenotype in wild-type recipients, demonstrating that NAFLD is a transmissible disease; two bacterial species (*Lachnospiraceae bacterium 609* and a relative of *Barnesiella intestinihominis*) were found to be dominant in mice which developed the NAFLD phenotype [4].

Gut dysbiosis is a modification of microbiome and promotes translocation of microorganisms and microbial products into the portal circulation (metabolic endotoxemia), which may activate pro-inflammatory cytokine production in the liver macrophages resulting in liver damage.

Gut microbiota alteration may also induce increased fermentation of carbohydrates to short-chain fatty acids (SCFAs) and subsequent stimulation of de novo synthesis of triglycerides in the liver. In NAFLD patients, gut dysbiosis also interferes with choline metabolism, which is required for very-low-density lipoprotein (VLDL) synthesis and hepatic lipid export.

In patients with NAFLD, several studies have demonstrated alterations of the gut microbiota, but interpretation of the results is not straightforward.

Microbiota analysis of patients with NAFLD showed an increase in the genus *Prevotella* and *Porphyromas* and reduction of *Bacteroidetes* compared to healthy controls. *Bacteroides* genus is correlated with NASH and a parallel decrease in *Prevotella* abundance. Diets enriched in fat, proteins of animal origin, and simple sugars, such as the Western diet, promote *Bacteroides* abundance, while an increase in *Prevotella* abundance is favored by a diet rich in fibers and vegetable carbohydrates. *Ruminococcus* genus has been positively associated with significant liver fibrosis (≥F2) in humans, and a correlation between the abundance of this genus and the development of metabolic impairment has been observed in animal models.

Alcohol production, due to the ability of *Ruminococcus* to ferment complex carbohydrates, may be responsible for further liver damage. Gut dysbiosis in NAFLD could also affect the conversion of primary bile acids into secondary bile acids. In particular, there is a higher level of *Enterobacteriaceae* (that could be potentially pathogenic) with lower *Lachnospiraceae*, and *Blautia* (with a 7α-dehydroxylating activity) abundances [5–7] (Fig. 5.1).

5.2.2 Intestinal Permeability

The intestinal microbiota plays a critical role in maintaining the integrity of the intestinal barrier and intestinal permeability, both factors being associated with the development of NAFLD/NASH. The role played by small intestinal bacterial overgrowth (SIBO) in NAFLD is established. The prevalence of SIBO in patients with NAFLD ranges between 50 and 78%. The causal relationship between SIBO and NASH can be identified in the alteration of intestinal permeability: the destruction of intestinal tight junctions leads to a greater exposure of the liver to pro-inflammatory agents such as lipopolysaccharide (LPS) and other bacterial metabolites, resulting in a dysregulation of inflammatory activity [8]. These data were confirmed by studies that directly linked the severity of the alteration of intestinal permeability with the degree of liver fibrosis. Furthermore, the demonstration of increased blood levels of LPS and inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)1-α in patients with NAFLD consolidated these data. Finally, the increase in serum inflammatory cytokines finds an equivalent in hepatic parenchyma, where, in the presence of SIBO, the liver expression of toll-like receptor 4 (TLR-4), release of IL-8, and of the TNF-α receptor p55 is increased [8].

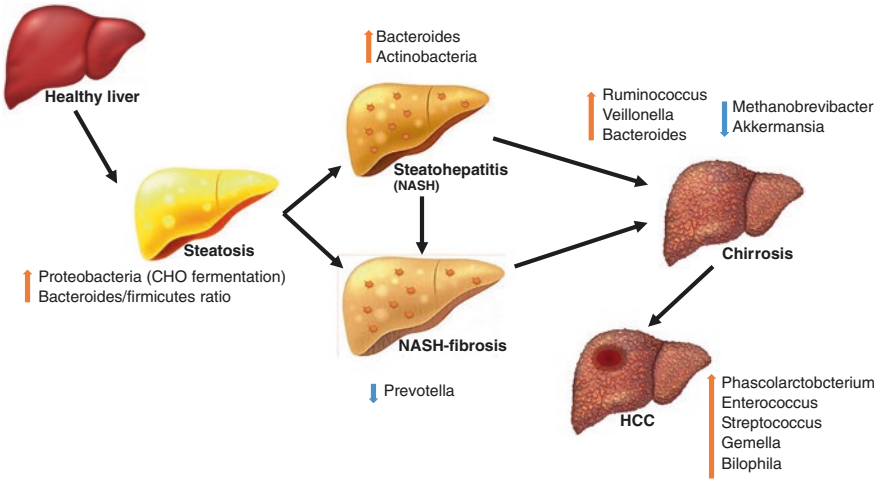

Fig. 5.1 Dysbiosis and NAFLD. Summary of evidence of microbiota signature in NAFLD at different stages of disease

NAFLD patients, and particularly those with NASH, are more likely to have increased intestinal permeability compared with healthy controls [9]. Patients with NAFLD have also a higher prevalence of SIBO, which enhances intestinal permeability. When intestinal permeability is increased, microorganisms and microorganism-derived molecules can translocate to the liver through the portal system, causing inflammation and hepatic injury.

5.2.3 Bile Acid Metabolism

Finally, a central role is played by bile acid metabolism. These components are involved in the maintenance of hepatic metabolism of glucose, cholesterol, and lipids, and it has already been demonstrated how the alteration of these pathways is an important factor in the development and progression of NAFLD [10]. The primary bile acids produced in humans are chenodeoxycholic acid (CDCA) and cholic acid (CA), which are conjugated with the amino acids glycine and to a lesser extent taurine before being excreted into bile [11] and released into the duodenum after a meal. Conjugated bile acids are then reabsorbed in the distal ileum by the apical sodium-dependent bile acid transporter (ASBT) and recirculated to the liver with portal blood (enterohepatic circulation). Bile acid synthesis is strictly regulated by negative feedback inhibition through the nuclear farnesoid X receptor (FXR), a transcription factor that is involved in the regulation of bile, glucose, and lipid metabolism [12, 13].

In humans, bile acid production and excretion in the biliary stream is modulated by the microbiota via SLC27A5 (Bile acyl:CoA synthetase), an enzyme responsible for conjugation. Microbial deconjugation of bile acid (i.e., removal of the glycine or taurine conjugate by bile salt hydrolases) prevents active reuptake from the small intestine via the ASBT. This reaction is performed by the bile acid hydrolase enzyme (BSH, bile salt hydrolase). This enzyme is expressed by important components of the gut microbiota, in particular *Lactobacilli* and *Bifidobacteria*, and confers them greater resistance to toxicity due to bile acids. Deconjugated primary bile acids that are not reuptaken enter the colon, where they are metabolized by the microbiota mainly through a 7α-dehydroxylation to yield the secondary bile acids lithocolic acid (from chenodeoxycholic acid) and desoxycholic acid (from cholic acid) [14] that are more hydrophobic and can thus be reabsorbed via passive diffusion, limiting bile acid loss through feces. A small proportion of deconjugated secondary bile acids is absorbed from the gut through passive diffusion, enters the enterohepatic circulation and possibly acts as signaling molecules [15]. Bacterial overgrowth shifts the balance between primary and secondary bile acids in favor of the latter, modifying subsequent enterohepatic cycling. Because of differences in the affinity of these two classes of bile acids for FXR, increased concentration of secondary bile acids modulates FXR-mediated hepatic synthesis of bile acids, leading to an overall increase in hepatic bile acid synthesis. These shifts have been associated with metabolic stress and host immune responses relevant to the progression of liver diseases [16].

Pool size and bile acid composition appear to be important factors in regulating the structure of intestinal microbiota. It is known that bile acids have direct antimicrobial effects, due to their amphipathic properties with detergent action on bacterial membranes, as well as indirect effects, mediated by activation of the nuclear bile acid receptor FXR [17].

These data are supported by the demonstration of gut microbiota modulation by oral administration of cholic acid in murine specimens. In particular, an increase in Firmicutes was observed at 98% (vs. 54% in the control population), with an increase in constriction to 70% (vs. 39% in controls); among these, the genus *Blautia*, which includes many species of *Clostridium* and *Ruminococcus* and other species with the ability to dehydroxylate primary bile acids to give secondary bile acids, increased to 55% compared to 8% of controls. A reduction in *Bacteroidetes* and *Actinobacteria* was also observed. Considering the *Firmicutes*' property of combining cholic acid with deoxycholic, with marked antimicrobial activity directed on the bacterial wall, it is possible to infer that reduced levels of bile acids in the intestinal lumen favor the proliferation of Gram-negative bacteria, among several pathogens. On the contrary, increased levels of bile acids in the intestine favor Gram-positive bacteria belonging to the *Firmicutes*, able to modify the primary bile acids of the host in secondary bile acids [15].

A new factor that plays a key role between gut microbiota and metabolism is FXR nuclear receptor. This role is supported by results of numerous studies that demonstrated a two-way relationship. Studies conducted on FXR knock-out mice (*Fxr*−/−) subjected to a high-fat diet show an increase in *Firmicutes* and relative reduction of *Bacteroidetes* compared to wild-type mice fed the same diet. In the same population, an alteration of the bile acid profile was also observed, showing higher levels of primary bile acids (β-muricolic acid and β-muricolic acid conjugated with taurine), indicating a reduced conversion to secondary bile acids [18]. These results suggest the importance of FXR in the metabolism of bile acids by the gut microbiota.

Bile acids can also act as hormones that influence the physiology and metabolism of the whole organism. Bile acids, metabolized by the microbiota in the intestine, partially enter the systemic circulation reaching a series of target organs in addition to the liver [19]. Bile acids act as hormones as they act as natural ligands for a large panel of receptors previously considered "orphans." These include other nuclear receptors in addition to FXR, such as Pregnane X Receptor (PXR), Vitamin D Receptor (VDR), and G-protein-coupled receptors such as Takeda G-protein-coupled receptor-5 (TGR5), Sphingosine-1-Phosphate Receptor 2 (S1PR2), and muscarinic receptor 2. Given that the bile acid profile of each subject depends on the type of diet and the composition of the intestinal microbiota, it is immediately understandable how varied is the way in which the signal transduction pathways regulated by these receptors can be modulated. Through metabolization of bile acids that will interact with these receptors, the microbiota is therefore able to influence different targets including intestinal physiology, lipid metabolism, glucose homeostasis (45), acid biosynthesis fat, the formation of brown adipose tissue, the regulation of the immune system, and the synthesis of bile acids.

5.3 The Bidirectional Link Between Diet and Microbiome

5.3.1 Microbiota Regulation of Substrate Metabolism

It has been known for a long time that consumption of dietary fiber is associated with a lower incidence of metabolic diseases [20]. Increasing evidence indicates that the gut microbiota may mediate the beneficial effects of fiber consumption on substrate metabolism, and several gut microbiota-derived metabolites may play a role.

Fermentation of dietary fiber produces SCFAs (mainly propionate, butyrate, and acetate) that can be absorbed into the circulation and serve as both microbiota-derived calories and signaling molecules [21]. SCFAs have different fates. Acetate is readily absorbed into the circulation, propionate is metabolized by the liver upon absorption, while the majority of butyrate is used locally by colonocytes as their primary fuel source. Short-chain fatty acids act as signaling molecules through several pathways, including serving as an energy substrate for colonocytes (mainly butyrate) [22, 23]; serving as a substrate for intestinal gluconeogenesis (propionate), which signals through the central nervous system to promote beneficial effects on food intake and glucose metabolism [24]; acting as histone deacetylase inhibitors (butyrate and acetate) to modulate gene transcription and expression [25, 26]; binding to G-protein-coupled free fatty acid receptors (FFARs) such as FFAR3 and FFAR2, which affect several important processes, including inflammation and enteroendocrine regulation [24, 27, 28]

Fermentable carbohydrates are the preferred substrate for the gut microbiota. However, the amount of fermentable carbohydrates that reaches the distal colon is low and, in the absence of their preferred substrate, gut bacteria in the distal colon switch from saccharolytic to protein fermentation, which yields a small amount of SCFAs and many compounds that negatively impact colonic health and metabolism [29]. Microbial proteolysis also produces branched chain amino acids (BCAA) and aromatic amino acids (AAA) that can be used as substrates by other gut microbes in complex cross-feeding pathways [30, 31]. Microbial metabolism of phosphatidylcholine, a phospholipid found in cheese, seafood eggs, and meat, and of L-carnitine, an amino acid that is abundant in red meat, produces high levels of trimethylamine (TMA). Once absorbed from the gut into the bloodstream, TMA circulates to the liver and is enzymatically oxidized to trimethylamine-Noxide (TMAO), a gut microbiota-derived metabolite that is significantly associated with the risk of type 2 diabetes (T2D), atherosclerosis, and incident major adverse cardiovascular events [32–34].

5.3.1.1 Glucose Metabolism

Several microbiota-derived metabolites are involved in the regulation of glucose metabolism, both through direct and indirect mechanisms.

Gut microbiota-derived SCFAs have been reported to stimulate insulin secretion via direct action on pancreatic β-cells [35] and by enhancing glucose-induced insulin secretion of glucagon-like peptide 1 (GLP-1) from enteroendocrine intestinal L cells [36]. The bile acid receptor TGR5 might have a role in energy homeostasis

by increasing energy expenditure in brown adipose tissue [36, 37] and in muscle, and by increasing GLP-1 release [36]. In pancreatic α cells, TGR5 activation shifts proglucagon processing from glucagon to GLP-1, while FXR and TGR5 activation in pancreatic β-cells promotes insulin secretion [38].

Finally, metabolites derived from proteolytic fermentation such as TMAO, BCAAs, hydrogen sulfide, p-cresol, and phenolic compounds, seem to be involved in the development of insulin resistance [29].

5.3.1.2 Lipid Metabolism

The gut microbiota modulates lipid metabolism and fat storage through different pathways. SFCAs produced by the gut microbiota are used by the liver as substrates to produce de novo lipids that are then stored in adipocytes [39]. Furthermore, acetate has been shown to exert anti-lipolytic effects. In overweight/obese men, colonic infusions of SCFA mixtures resulting in increased circulating acetate attenuated fasting free glycerol concentrations (which reflect lipolysis) [40]. Similarly, intravenous acetate was shown to reduce fasting FFAs in healthy volunteers with or without insulin resistance [41]. Other studies in overweight/obese subjects indicate that acute infusions of either acetate or as SCFA mixtures of acetate, propionate, and butyrate in the distal colon increased fasting lipid oxidation and resting energy expenditure [40, 42]. In addition, acute oral propionate administration was shown to raise resting energy expenditure and fasting lipid oxidation, independent of insulin and glucose levels and sympathetic nervous system activity in healthy volunteers [43]. Overall, increased lipid-buffering capacity in adipose tissue and reduced lipolysis may lead to reduced circulating FFAs, which in turn may result in improvements in insulin sensitivity and reduced ectopic fat accumulation.

Bile acids may also influence lipid metabolism. Via activation of the bile acid receptor FXR in the liver, bile acids modulate the absorption of glucose, decrease glycolysis and lipogenesis, hence decreasing VLDL-TG production [38]. Finally, BCAAs, which may serve as substrates for de novo lipogenesis in liver, may also promote adipose tissue expansion [44].

5.3.2 Diet-Induced Alterations in Gut Microbiota and Their Impact on Metabolic Processes

Extensive evidence exists that diet modulates the composition and function of gut microbiota in humans and other mammals [45–52], and gut microbiota composition [53] and diversity [46, 53–56] differ in populations with different dietary habits. Human gut microbiota responds rapidly to large changes in diet, as quick as within 1–2 days [45, 47]. However, long-term dietary habits are a dominant force in shaping the composition of gut microbiota. Long-term dietary trends are linked to characteristics of microbiota composition [47, 52, 57, 58], and it appears that an individual's gut microbiota consists of both a stable "core" and less stable bacterial taxa [47].

5.3.2.1 Effect of Diet on Gut Microbiota

Diet is a primary modulator of gut microbiota composition and function. In particular, dietary carbohydrates appear to be the main driver of colonic fermentation, as suggested by a study that compared three weight loss diets with varying protein and carbohydrate intakes in overweight/obese adult healthy men, finding that most fecal metabolites were significantly associated with carbohydrate intake [59]. Changes in macronutrient components of diet may significantly impact microbial metabolic activity. Animal-based diets rich in fat and protein associate with increased fecal bile acid concentration, and significant increases in the abundance of microbial DNA and RNA encoding sulfite reductases [45], an enzyme necessary to reduce sulfite to hydrogen sulfide (a compound associated with bowel inflammation) [60]. Dietary fat has been shown to increase circulating endotoxins in both mice and humans [61, 62], which could trigger and sustain the low-grade inflammation that characterizes obesity and associated metabolic disturbances [62–64]. The type of dietary fat is also important in determining gut microbiota responses. Saturated and unsaturated fats have significantly different effects on the gut microbiota. Animal studies demonstrated that the altered microbiota resulting from a diet rich in saturated fats may mediate white adipose tissue inflammation and impaired insulin sensitivity, whereas the microbiota of mice fed unsaturated fats (fish oil) provide protection against lard-diet-induced adiposity and inflammation [65]. Furthermore, mice fed fish oil had increased levels of taxa from the genera *Lactobacillus* and *Akkermansia muciniphila*, whereas mice fed lard had increased levels of taxa related to *Bilophila* [65], which may also increase in humans consuming diets rich in saturated fats of animal origin [45].

Acute consumption of the prebiotic inulin (a fermentable fiber) improves fat oxidation and promotes SCFA production in overweight/obese men as compared with placebo (maltodextrin, a digestible carbohydrate) [66], and may reduce secretion of the hunger hormone ghrelin in lean and overweight/obese people [67]. Besides macronutrients, other dietary elements may affect gut microbiota. Emulsifiers and artificial sweeteners have been shown to be involved in the development of metabolic syndrome features through their modulation of the microbiota in mice [68, 69].

Thus, diet appears to exert variable effects on the host metabolism through the microbiota, which may have a role in determining an individual's response to a specific diet [70]. However, changes in diet composition can have a highly variable effect on different subjects, due to the unique nature of an individual's gut microbiota [48, 50].

Weight loss interventions may reduce SCFA concentrations in overweight or obese adults, particularly if carbohydrate intake is low [71]. Other diet-induced changes in gut microbiota metabolic processes affecting host metabolism include variations in the levels of circulating gut microbiome-related metabolites, such as TMAO. A greater decrease in TMAO during weight loss was associated with greater improvements in insulin sensitivity at 6 months in overweight and obese adults [72]. TMA metabolism exemplifies the interaction between diet and microbiota.

TMA-producing microbiota generate the metabolite only when the diet includes compounds that contain TMA (e.g., choline, carnitine, betaine), and some microbiota (e.g., those of vegans) are poor producers of TMA [57], even when TMA precursors are transiently consumed. Diets in which the major protein source is red meat result in substantial increases in fasting plasma and urine TMAO levels, as compared with either white meat or non-meat sources [73]. Furthermore, overweight and obese individuals with higher levels of TMAO have been reported to consume diets low in carbohydrates and high in fat [72]. Thus, the microbiota changes to adapt to specific macronutrients, and diets rich in protein and saturated fat may shift the gut microbiota composition toward a metabolically unfavorable profile.

5.3.3 Influence of Gut Microbiota on Hormones Involved in Metabolic Processes

Gut hormones are secreted by specialized enteroendocrine (EE) cells within the mucosal lining of the gut that are involved in the regulation of a range of metabolic processes. There is evidence that the gut microbiota influence the secretion of gut hormones, both directly and indirectly [74].

GLP-1. GLP-1 is an incretin hormone released by the intestinal epithelial endocrine L-cells in response to nutrients such as glucose to enhance glucose-dependent insulin secretion and inhibit glucagon secretion from the pancreas. In addition, GLP-1 exerts inhibitory effects on food intake and gastric emptying [75]. Preclinical studies suggest that butyrate may improve glucose metabolism by inducing GLP-1 secretion from the colonic L cells [76, 77]. Consistently, total levels of propionate and acetate were inversely correlated with insulin resistance in individuals with T2D [78]. By modulating gut hormones such as GLP-1 and peptide tyrosine tyrosine (PYY), which exerts anorexigenic effects by inhibiting neuropeptide Y and activation of satiety-inducing pathways in the hypothalamic arcuate nucleus [79, 80], the gut microbiota may also affect appetite [81].

Serotonin. Gut-derived serotonin 5-HT is involved in metabolic regulation through interactions with key metabolic target tissues/organs, namely the GI tract, pancreas, liver, adipose tissue, and bone [82]. Of note, altered gut-derived 5-HT is related to both obesity and altered glucose metabolism [83]. Absence of 5-HT, through genetic or pharmacological blockade of peripheral of tryptophan hydroxylase 1 (TPH1), i.e., the rate-limiting enzyme for 5-HT synthesis in the gut, protects against the development of metabolic syndrome in mice fed a high-fat diet [84]. Gut microbiota play an important role in regulating both the synthesis and secretion of 5-HT by intestinal enterochromaffin (EC) cells [85]. Secondary bile acids such as DCA may also stimulate the secretion of 5-HT via activation of the bile acid receptor TGR5. Increased plasma 5-HT can negatively affect metabolism by reducing insulin sensitivity and glucose tolerance, increasing intestinal fat absorption and circulating FFA, and increasing systemic inflammation [82].

5.3.4 Alterations of Macronutrient Processing and Impact on Metabolic Processes

Metagenomic studies have shown associations between improved metabolic health and a relatively high microbiota gene content and microbial diversity [48, 86, 87], whereas obesity appears to be associated with reduced gut microbiota diversity [86, 87].

In lean individuals, gut microbiota was shown to be associated with an increased production of SCFAs, whereas the microbiota of obese individuals had an increased abundance of genes that are involved in the biosynthesis of BCAA, which in turn are associated with impaired insulin sensitivity [88]. Consistently, the gut microbiota of individuals with the metabolic syndrome is characterized by reduced abundance of bacterial species with saccharolytic activity [89] that might lead to a reduction in the production of beneficial SCFAs such as propionate and acetate. In turn, a reduction in acetate levels in the gut may decrease the abundance of beneficial bacteria such as *Faecalibacterium prausnitzii* and *Eubacterium rectale*, which consume acetate and produce butyrate, and may also directly degrade carbohydrates to produce butyrate [90]

Evidence that the gut microbiota influences responses to food is provided by a study that used continuous blood-glucose monitoring to follow postprandial glycemic responses in 800 participants [70], individual responses to identical foods were highly variable. However, using a machine-learning algorithm, integrating blood parameters, dietary habits, anthropometrics, physical activity, and gut microbiota, the response of an individual to a given food could be predicted. Gut microbial composition may also affect the response to dietary interventions. *Akkermansia muciniphila*, a mucin-degrading bacterium, plays a role in maintaining gut barrier health by modulating the translocation of microbial molecules across the gut [91]. Subjects with higher gene richness and *Akkermansia muciniphila* abundance at baseline exhibit greater improvements in glucose homeostasis and LDL cholesterol after a weight loss intervention [92]. Further evidence that gut microbiota mediates the effects of diet comes from a lifestyle intervention study that compared overweight/obese individuals who achieved at least 5% and those who achieved less than 5% weight loss, demonstrating that increased abundance of *Phascolarctobacterium* at baseline was associated with successful weight loss, whereas increased abundance of *Dialister* was associated with <5% weight loss [93].

Although SCFAs appear to exert metabolic benefits acting as regulatory molecules [24], the metabolic characteristics of the host appear to influence their effect on metabolic processes. As an example, oral administration of butyrate for 4 weeks resulted in significant improvements in peripheral and hepatic insulin sensitivity in lean, but not in metabolic syndrome subjects [42].

Finally, in contrast with the large body of evidence supporting a beneficial effect of SCFAs on host metabolism, it has also been hypothesized that gut microbiota composition of obese individuals may determine increased energy harvesting [94]. Pathways that generate SCFAs were found to be enriched in metagenomic studies of obesity, and increased fecal SCFAs have been reported in obese compared with lean

individuals [94–96], suggesting enhanced energy harvest contributing to obesity or, possibly, altered SCFA handling and/or SCFA resistance in obese subjects. In fact, it is not known whether fecal concentrations of SCFAs mirror SCFA production, and it is also possible that increased fecal SCFA content reflects reduced intestinal absorption of SCFAs.

5.4 Role of Gut–Liver Axis in the Onset and Progression of Liver Damage in NAFLD/NASH

5.4.1 Dietary Factors

Diets rich in saturated fats, fructose, and cholesterol alter the gut microbiota and intestinal barrier function favoring onset and progression of NAFLD. Patients with NAFLD are reported to engage in an excessive consumption of total energy, refined carbohydrates (including fructose), saturated fats and cholesterol with an insufficient intake of polyunsaturated fats, fibers, and antioxidants (vitamin C and vitamin E) [97, 98].

Diet modifies the gut microbiota, even very rapidly [99]. In the seminal study of Fava and coll [100]. a high-carbohydrate diet, irrespective of glycemic index, could modulate human fecal saccharolytic bacteria, including *Bacteroides* and *Bifidobacteria*. Conversely, high-fat diets did not affect individual bacterial population but reduced total bacteria number; fecal excretion of SCFA is increased, suggesting a compensatory mechanism to eliminate excess dietary energy.

Certain dietary components, in particular saturated fats and fructose, can alter gut barrier integrity leading to increased gut permeability to bacteria and bacteria-derived products, including endotoxin LPS [101]. Many studies conducted in animal models of NAFLD demonstrated that a high fat-diet or a high-fructose diet resulted in elevated circulating LPS levels in parallel with the significantly increased hepatic fat accumulation and a significant reduction in the expression of the intestinal occludin protein [64, 102]. Consistent with the animal models, several recent studies have reported elevated levels of blood endotoxin in pediatric patients with NASH [103] and also in healthy individuals after high-fat meals or high-fructose drinks [104].

5.4.2 Metabolic Endotoxemia and Low-Grade Inflammation

Intestinal dysbiosis (anomalous or imbalanced gut microbial composition) and increased intestinal permeability lead to translocation of microorganisms and microbial products, including cell wall components (endotoxins from Gramnegative bacteria and βglucan from fungi) and DNA, together referred to as MicrobialAssociated Molecular Patterns (MAMPs) [105]. After reaching the liver through portal circulation, MAMPs induce localized inflammation through PatternRecognition Receptors (PRRs) on Kupffer cells and hepatic stellate cells. Activation of TLR4 (activated by LPS), TLR9 (activated by methylated DNA), and TLR2 (activated by Grampositive

bacteria) are the primary drivers of immune response in liver disease. TLR signaling in Kupffer cells in the liver activates a downstream proinflammatory cascade, leading to Myeloid Differentiation primary response protein MYD88mediated activation of Nuclear FactorκB (NFκB). Additionally, TLR4 signaling also promotes fibrosis by downregulating BMP and activin membrane-bound inhibitor homologue (a decoy receptor for transforming growth factor β in hepatic stellate cells). These steps lead to expression of inflammatory cytokines, oxidative, and endoplasmic reticulum stress and subsequent liver damage [106]. TLRs receptors are able to recognize bacterial particles (PAMPs). In particular, TLR-4 is a receptor for LPS, TLR-9 for particles of bacterial DNA containing unmethylated CpG motifs; TLR-2 binds components of gram-positive bacterial cell walls, such as peptidoglycan and lipoteichoic acid; TLR-5 recognizes flagellin, the major protein constituent of bacterial flagella. In models of experimental acute colitis that the presence of *Clostridium Butyricum*, via the production of pro-inflammatory components such as IL-10 by intestinal macrophages, leads to activation of TLR-2. This last receptor has an ambiguous behavior: the administration of probiotics has led to the production of anti-inflammatory modulators always activating the TLR-2. These studies have shown that the activity of these receptors can be modulated by the bacterial component with which they come into contact. This innovative concept opens the way to various modulation possibilities through the management of the gut microbiota [107, 108].

5.4.3 Production of Endogenous Ethanol

Even in pediatric NAFLD patients who are completely abstinent, increased concentrations of blood ethanol have been reported [109]. Elevated representation of *Escherichia* (alcohol-producing bacteria) was observed in parallel with the increased blood alcohol concentration in NASH patients, suggesting a novel mechanism for the pathogenesis of NASH. Thus, gut microbiota enriched in alcohol-producing bacteria (e.g., *E. coli, Ruminococcus*) constantly produce more alcohol, which is known to play an important role in the disruption of intestinal tight junctions causing hepatic oxidative stress and inducing liver inflammation [110].

5.4.4 Free Fatty Acid Metabolism

In NAFLD patients, high-carbohydrate diet may induce saccharolytic bacteria, including Bacteroides and Bifidobacteria, which induce increased fermentation of carbohydrates to SCFAs and subsequent stimulation of de novo synthesis of triglycerides in the liver. In animal models fed a high-fat diet, the increased flow of free fatty acids to the liver trough portal circulation is associated with liver damage via increased oxidative stress [111].

5.4.5 Modulation of Endocannabinoid System

Gut microbiota modulate the intestinal endocannabinoid system tone, which in turn regulates gut permeability and plasma LPS levels [112]. In animal models of obesity, the endocannabinoid system tone is found to be overactive in the colon and in adipose tissue. This phenomenon is associated with the development of gut permeability, metabolic endotoxemia, and with altered adipose tissue metabolism (adipogenesis). In animal models, prebiotic (oligofructose) treatment changes the gut microbiota composition, leading to a decreased endocannabinoid system tone in colon and adipose tissue, thereby counteracting gut permeability and metabolic endotoxemia [113].

5.4.6 Modulation of Choline Metabolism

In patients with intestinal dysbiosis, choline metabolism is altered. In normal conditions, the macronutrient choline is metabolized into phosphatidylcholine (lecithin) by the host, which assists in excretion of VLDL from the liver. In conditions of intestinal dysbiosis, choline can be converted to TMA by intestinal bacteria; TMA is transported to the liver through the portal circulation where it is converted to TMAO. Increased systemic circulation of TMAO is concomitant with reduced levels of hostproduced phosphatidylcholine. In these conditions, synthesis of VLDL is impaired and triglycerides accumulate into the liver. In a Chinese case-control study, circulating TMAO levels were inversely associated with both presence and severity of NAFLD [114].

Choline is also an important metabolite that influences numerous physiological processes in the liver, modulating lipid metabolism and enterohepatic bile acid circulation [19]. Besides the already known genetic and dietary factors, recent studies have also identified the gut microbiota as a factor able to modulate choline production. In particular, intestinal bacteria enzymatically convert food-derived choline to dimethylamine (DMA) and TMA. These products then enter the enterohepatic circulation through intestinal microvilli reaching the portal circulation, where they exert a pro-inflammatory action. The importance of gut microbiota activity in this process has been confirmed by studies on germ-free mice, where TMA production is zeroed [115, 116].

5.5 Therapeutic Perspectives from Gut–Liver Axis

Lifestyle changes are the core of NAFLD therapy [117]. The association between improvements derived from the modulation of diet and physical activity, leading to a refinement of the metabolic system and, in particular, of the liver function profile, is well established. At present, no pharmacological treatment has proven effective.

Numerous studies have been carried out with the aim of establishing how the action on the intestinal flora can lead to optimal results in terms of metabolic structure, with poor side effects.

The most commonly used strategies of manipulating gut microbiota include the use of probiotic, prebiotic, or synbiotic supplements, or antibiotic treatment [118].

5.5.1 Prebiotics

The prebiotics, identified by Marcel Roberfroid in 1995, are substrates that are used selectively by a host microorganism to produce a health benefit, as defined in 2016 by the International Scientific Association for Probiotics and Prebiotics (ISAPP).

The main mode of action of these substrates is the modulation of the gut microbiota, making available nutrients that can favor the growth of certain bacterial species [119]. There are numerous studies in animals and humans which highlight the potential of prebiotics for the treatment of NAFLD, having an important activity on body weight reduction. These studies have indeed demonstrated an increase in GLP-1 and PYY, thus affecting satiety.

Besides acting directly on metabolic control, the main mode of action is on the gut microbiota. For example, through the stimulation of the hormone glucagon-peptide-2, prebiotics are able to reduce the levels of intestinal permeability, thus acting on the bacterial translocation.

The beneficial effects on NAFLD are supported by studies that have shown that prolonged treatment with prebiotic (fructooligosaccharide) plus probiotic (*Bifidobacterium longum*) leads to reduction of serum AST, endotoxin, and hepatic steatosis in NASH patients.

The efficacy of this treatment in the reduction of hepatic lipogenesis and of the blood levels of triglycerides has been demonstrated. It has also been shown that through the manipulation of the intestinal flora, there is an increase in SCFAs, in particular acetate and propionate [120].

Therefore, prebiotics and synbiotics may hold promise for the treatment of patients with NAFLD in clinical practice.

5.5.2 Probiotics

Probiotics are defined as live bacteria or yeasts of human origin that provide health benefits if consumed by modulating the intestinal microbiota. Based on the awareness of the role of the microbiota in metabolic diseases, several studies have been conducted in this direction [121].

Changes in the intestinal microbiota through the use of probiotics seem to be able to interfere with the evolution of NAFLD by limiting its progression to fibrosis in numerous animal models [122]. Administration of probiotics can normalize intestinal permeability, reducing it and minimizing endotoxins with its

inflammatory consequences. A probiotic formulation called VSL # 3 (*Streptococcus salivarius* subsp., *Thermophilus, Bifidobacterium [B. breve, B. infantis, B. longum], Lactobacillus acidophilus, L. plantarum, L. casei*, and *L. delbrueckii* subsp. *Bulgaricus*) has been widely studied in the treatment of NAFLD. In a mouse model of genetically determined dyslipidemia (Apo-E knock-out mice that do not develop NASH-like hepatopathy when placed on a standard diet), it was observed that when intestinal inflammation was induced after treatment with dextran sodium sulfate (DSS), destruction of the intestinal tight junctions and an increase in intestinal permeability were determined. Those events lead to an evolution of steatotic liver disease toward typical NASH. This evolution was interrupted when therapeutic administration was performed with VSL # 3.

In experimental models of NAFLD obtained by a high-fructose diet, administration of *Lactobacillus rhamnosus* GG was shown as a protective agent, preventing the development of NAFLD by reducing the levels of duodenal inhibitor of kappa B (IκB), reducing activation of the TLR-4 signaling cascade, and increasing peroxisome proliferator-activated receptor gamma (PPAR-γ) activity. Furthermore, treatment based on *Lactobacillus rhamnosus* GG has been demonstrated to lead to a reduction in endotoxemia, TNF-α levels, IL-8R, and IL-1b mRNA expression in the liver. In addition, LGG decreased cholesterol levels, mediated by suppression of FXR and fibroblast growth factor 15 signaling. Consensually also changes in the microbiota have been detected, where the population of *Enterobacteriaceae* was reduced and the relative abundance of *Clostridiales* Family XIV was increased.

Contrary to expectations, the administration of *Lactobacillus acidophilus* has no effect on liver function or alteration of intestinal permeability. This result, however unexpected, is of fundamental importance: the identification of species that can become a specific therapeutic actor for pathology will be one of the challenges of the future [123, 124].

Probiotics also act at the level of the TLRs, modulating the TLR4/LPS response. The prolonged treatment based on a combination of *Acetobacterium, Bifidobacteurum, Lactobacillus, Lactococcus, Propionibacterium*, associated with drugs capable of modulating the glycemic response, did not in fact improve the degree of steatosis and the indices of liver function, and also led to a reduction of IL-1β, TNF-a, IL-6, IL-8, and interferon (IFN)-γ [114].

These scientific evidences can therefore confirm not only the role of the intestinal flora in the development of the disease, but, above all, they open new therapeutic opportunities, offering handy and safe instruments for clinical practice. In order to consolidate these data, further clarifications on the pathophysiological mechanism are still needed, in order to define more specific and targeted treatments in view of the vastness of probiotics currently available [115]. From the point of view of clinical practice, it is also necessary to define instrumental laboratory indexes that can monitor the response to treatment.

In order to modulate the gut microbiota, the drugs whose mechanism of action is best known are antibiotics, in the face of more consistent systemic effects compared to the overdescribed possibilities.

5.5.3 Antibiotics

The use of non-absorbable antibiotics such as rifaximin is already long established in the treatment of hepatic encephalopathy (HE). This use is supported by the underlying pathophysiological mechanism as bacterial translocation and bacterial products play an important role in the development of HE. Furthermore, the efficacy of antibiotic therapy in liver diseases has been amply demonstrated by numerous studies. Since these are drugs with considerable systemic effects, the agent, the therapeutic target, and the timing of therapy must be carefully identified. In fact, not all antibiotics tested show beneficial effects on the host and disease progression. Numerous studies have evaluated the effect of antimicrobial therapy on liver disease [125].

The effects of polymyxin B and neomycin have been studied in animal models of liver disease: this treatment, in combination with a fructose supplement, showed a reduction in hepatic lipid accumulation. Treatment with neomycin alone showed that the air exhaled by the ob/ob mice contained a high concentration of alcohol compared to that of the control mice, indicating that the changes induced by neomycin in the intestinal microbiota decreased alcohol systemic concentrations. Another antibiotic molecule such as cidomycin, evaluated on murine models of NASH, has been shown to have a beneficial effect on hepatic function indices (ALT and AST) and inflammatory factors (TNF-α).

Another study suggested that a new combination treatment of capsaicin and antibiotics works synergistically to mitigate HFD-induced obesity, steatosis, and metabolic disease [126].

These assessments performed on mouse models have subsequently found confirmation in studies conducted on humans. In particular, it has been shown that the administration of rifaximin decreases the circulating levels of endotoxin, IL-10, ferritin, and AST in patients with NAFLD [127]. An ongoing study showed that treatment with soltromycin leads to a reduction in the NAFLD activity score and ALT levels.

Obviously, although the use of antibiotics is already approved for the management of HE and numerous studies support its use in the management of metabolic disease, it is important to evaluate the effect of the molecule well on the entire microbial population, choosing antibiotic molecules with a low potential to create resistance and poor systemic adverse effects.

5.5.4 Fecal Microbiota Transplantation (FMT)

Fecal microbiota transplantation is the process by which the feces of a healthy donor are collected and transplanted onto a sick individual. Similar to organ transplantation, the goal of FMT is to improve the health of the transplant recipient. The FMT process not only transplants live and dead microorganisms but also small food particles, small and large intestine cells, and metabolic products of bacteria. The mechanism by which FMT is effective is not well defined for most diseases. However, it is believed that the benefits derived from FMT are due to an increase in

favorable microbes, an increase in microbial diversity, and stimulation of mucosal immunity [128].

In humans, FMT is most commonly used as a therapeutic tool for recurrent *Clostridium difficile* infections that do not respond to antimicrobial treatment. However, FMT has recently been recognized as a potential therapy for a wide range of other diseases in humans, such as irritable bowel syndrome, colonization of antimicrobial resistant pathogens, metabolic syndrome, and insulin resistance [129].

Obviously, this therapeutic option provides a complete distortion of the intestinal flora. It is also an invasive procedure with possible significant adverse events, mostly infectious. Therefore studies on animal models are still needed to ascertain their effectiveness and safety in this setting [15].

As already mentioned, the liver produces BA from cholesterol, which is then transformed in the intestine, where it is metabolized by some members of the microbiota. Approximately 95% of the BA secreted through the bile duct is reabsorbed by the intestine (most commonly in the distal ileum) as BA conjugates through the action of the sodium-dependent apical BA transporter and are then returned to the liver via the portal vein, from which are again secreted. This enterohepatic circulation is repeated about six times a day. The components of the gut microbiota become part of this mechanism through the enzyme hydrolase, avoiding direct activity on the removal of the glycine/taurin conjugates, thus preventing the active reuptake from the small intestine [17].

The definition of the activity of FXR and TGR5 has promoted new research on the modulation of these receptors. The gut microbiota has direct interaction, through the production of bile acids, thus having a significant effect on the modulation of glucose and lipid metabolism. In this regard, a phase III study (NCT02548351) is underway, studying the effects of obeticholic acid, an FXR agonist, in patients with NASH in terms of histological and clinical evaluation. The study is still in the enrolment phase; therefore, the outcomes of primary end points are not known.

The modulation of the microbiota to rich populations of the enzyme hydrolase showed an increase in deconjugation and fecal excretion of the BA. Consensually, downregulation of the enterohepatic FXR/Fgf15 axis leading to a reduction in the levels of neosynthesis of hepatic BA and BA reuptake levels has been demonstrated [115].

In this context it has also been shown that FXR agonists do not lead to a beneficial effect only on hepatic metabolism, but they also act upstream, improving the state of the intestinal barrier and thus reducing permeability.

However, a liver agonist produced in the liver was found to constitutively stimulate FXR in the ileum of obese mice, thus leading to higher levels of ceramide production. Ceramides is able to activate lipid toxicity in the liver by increasing endoplasmic reticulum stress, leading to higher levels of fatty acid synthesis. Therefore, inhibition of the FXR/ceramide axis, with the action of gut microbiota, mediates the development of NAFLD, suggesting that FXR in the intestine is a potential therapeutic target in the treatment of NAFLD [115].

Absorbents are another therapeutic option for NAFLD. These compounds are not absorbable in the intestine, but work by binding to toxins or PAMPs. Among

these, a new synthetic activated carbon, Yaq-001 (Yaqrit Ltd.), was developed with carefully studied porosity (both macroporous and microporous) in order to have specific action on bacterial translocation, leading to a reduction in endotoxemia. Furthermore, Yaq-001 also has a binding action on other intestinal metabolites, such as acetaldehyde, ammonia, dimethylarginine, and hydrophobic BA, products derived from bacteria and proinflammatory cytokines, including TNF-α and IL-6. As Yaq-001 is regulated as a device in Europe, a safety and efficacy study is underway. This study should lead to a CE mark (CARBALIVE-SAFETY), followed by the PREVENT-ACLF and TREAT-NAFLD clinical studies to study the safety and efficacy of Yaq-001, respectively, for decompensated cirrhosis and NAFLD [115].

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Mechanisms of Fibrogenesis in NASH

6

Mirella Pastore, Alessandra Gentilini, and Fabio Marra

6.1 Introduction

NAFLD includes a wide spectrum of conditions, ranging from simple accumulation of fat ("fatty liver" or steatosis), to steatohepatitis (NASH, 7–30% of patients), fibrosis, and cirrhosis [1]. Progression of NASH can lead to end-stage liver disease and hepatocellular carcinoma (HCC), conditions associated with increased morbidity and mortality. The mechanisms underlying the development and progression of NAFLD are complex and multifactorial. A multiple-hit hypothesis has now substituted the outdated two-hit hypothesis for the progression of NAFLD, where accumulation of lipids in the liver (steatosis) represented the first hit, followed by a second hit leading to NASH, represented by oxidative stress, lipid peroxidation, and mitochondrial dysfunction [2, 3]. Indeed, accumulating information indicates that multiple hits occur at the same time and promote liver inflammation and fibrogenesis [4]. Activation of inflammatory and fibrogenic pathways within the liver, in association with signals deriving from extrahepatic sites, including the adipose tissue and the gut, contribute to generate signals potentially leading to NAFLD progression [5]. The deposition of fat in the liver is the result of imbalance between the rate of influx and removal of fatty acids, leading to accumulation of triglycerides, a system that protects hepatocytes from lipotoxicity resulting from excessive influx of free fatty acids (FFAs) and other lipotoxic agents [6, 7]. Most of the FFAs accumulated as triglycerides originate from increased lipolysis in peripheral tissues as a result of adipose tissue insulin resistance (IR), leading to lipogenesis favored by hyperinsulinemia and dietary fat [4]. These events cause a series of processes responsible for inflammation and activation of the fibrogenic process, including

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cytokine expression, apoptosis, oxidative stress (OS), lipid peroxidation, and mitochondrial dysfunction. These pathophysiologic components underly the major histopathological characteristics of NASH, namely hepatocellular ballooning, fibrosis, and cirrhosis [4].

6.2 Basic Pathophysiology of Liver Fibrosis

The fibrogenic process is a reparative response characterized by deposition of excessive fibrillar extracellular matrix tissue, resulting in progressive architectural remodeling in nearly all tissues and organs [8]. In chronic liver diseases, the fibrogenic process initiates following parenchymal cell injury, mediated by the action of various damaging agents that cause cell death (necrosis, apoptosis, necroptosis, pyroptosis, ferroptosis, and others) [8]. The consequent tissue injury is associated with inflammation, whereby immune cells (mainly resident macrophages) become activated, and several types of leukocytes are recruited to the site of injury. Local and recruited immune cells produce a variety of soluble mediators including proinflammatory cytokines and chemokines that lead to the activation of mesenchymal cells, which produce extracellular matrix (ECM) [8, 9]. In addition, the complement system becomes activated, attracting phagocytes that are stimulated by harmful or damaged material [9]. Moreover, the coagulation cascade and the fibrinolytic system allow the reabsorption of thrombotic material and may trigger fibrogenesis. In an acute setting, when these biological activities are sufficient for removal of injurious agent and wound healing, the soluble mediators are removed, and tissue homeostasis is restored. Nevertheless, if these initial responses are insufficient to eliminate the damaging stimuli, inflammation persists and immune cells (e.g., macrophages and T lymphocytes) are prompted to produce cytokines and other molecules that cause long-lasting damage [8]. These processes perpetuate parenchymal cell death, resulting in loss of membrane integrity and release of cell death-related products and profibrogenic mediators in the surrounding milieu, which in turn stimulate the activation of profibrogenic cells. In this scenario, cells producing ECM components increase in number and become excessively activated, leading to an excess of ECM with consequent scar formation and destruction of the normal organ architecture [10, 11].

The liver is responsible for protein, carbohydrate and lipid metabolism, elimination of drugs and toxins from the blood, and regulation of immune responses [11]. The hepatic parenchyma is organized in functional units consisting of hepatocytes, endothelial cells, Kupffer cells, hepatic stellate cells (HSCs), and bile duct cells [12]. All hepatic cells are susceptible to numerous insults and participate in various pathophysiologic mechanisms following liver damage [13]. On the other hand, the liver has a considerable regenerative potential, as shown by the ability to regulate its growth and mass after hepatectomy and recover after acute liver injury [14].

Chronic damage leads to the development of liver fibrosis with accumulation of ECM components, mostly fibrillar collagens, fibronectin, and proteoglycans, which are major players in the alteration of the hepatic architecture [15]. Like in other

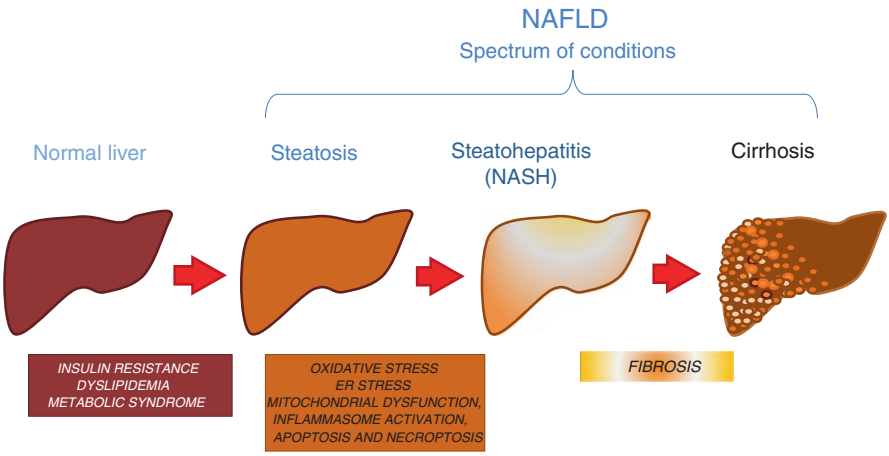

Fig. 6.1 Evolution of NAFLD to NASH and to possible end-stage liver disease

chronic liver diseases, fibrogenesis occurs in NAFLD, and particularly in NASH, where it is associated with a higher risk of progression to advanced disease and cirrhosis, and to all-cause mortality [16, 17]. There are some peculiarities of fibrosis in NASH (Fig. 6.1), compared to the one observed in chronic viral hepatitis or in other forms of chronic liver disease. Deposition of ECM in NASH occurs predominantly in the perisinusoidal space, in zone 3 of the hepatic acinus, similar to what is observed in alcoholic liver disease [18]. This pattern of fibrosis formation has been defined as pericellular, perisinusoidal, and "chickenwire," but eventually collagen deposition also occurs in periportal area, leading to the development of bridging fibrosis and cirrhosis. The different aspects of fibrosis in NASH are associated, at least in part, with different pathogenetic factors related to the presence of the metabolic syndrome and its related disturbances [19]. As it will appear clear in this chapter, there is a relevant contribution of extrahepatic tissues to the pathogenesis of NASH fibrosis, more evident than in other conditions of chronic liver disease. Here we will discuss the major pathophysiologic mechanisms underlying the development of fibrosis in NASH, with an emphasis on the therapeutic targets highlighted by recent research.

6.3 Cellular Interplay in NAFLD Pathogenesis

The mechanisms underlying liver fibrosis are complex and involve the interplay of multiple factors. A key role is played by the cross talk between various liver-resident and infiltrating cellular subsets, which produce and secrete different soluble mediators (cytokines and chemokines) and are further modulated by the chemical and biological properties of the etiological agent [20]. In most cases, tissue injury induces an inflammatory response involving the local vascular system, immune cells, and release of endocrine mediators. In this context, non-parenchymal cells

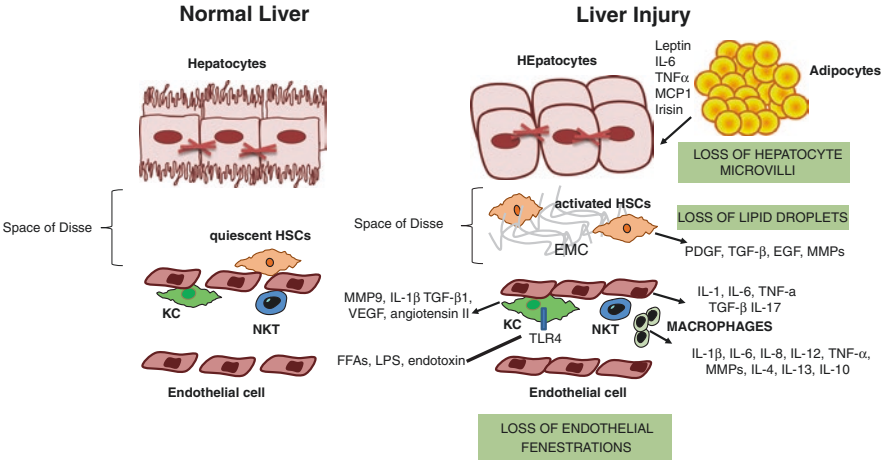

Fig. 6.2 Schematic representation of cellular and molecular mechanisms that regulate liver fibrosis in NAFLD. Injurious agents cause parenchymal cell destruction that induces the activation of local immune cells (mainly resident macrophages) and the recruitment of several types of blood cells. Local and recruited immune cells produce a wide variety of soluble mediators including proinflammatory cytokines and chemokines that lead to the activation of mesenchymal cells, which produce ECM. The accumulation of ECM in space of Disse activates quiescent HSCs leading to the loss of hepatocyte microvilli and disappearance of endothelial fenestrations. These architectural changes impair transport of solutes from the sinusoid to the hepatocytes, further contributing to the hepatocyte damage. *ECM* extracellular matrix, *HSCs* hepatic stellate cells, *MMP* metalloproteinases, *TGF-β* transforming growth factor, *PDGF* platelet-derived growth factor, *TLR* toll-like receptor, *TNF-α* tumor necrosis factor α, *MPC1* monocyte chemoattractant protein 1

(endothelial cells and HSCs) and resident or recruited immune cells (macrophages, lymphocytes, dendritic cells, and mast cells) with specialized surface receptors secrete a variety of inflammatory and profibrogenic mediators. These factors, especially cytokines and chemokines, such as tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β), as well as reactive oxygen species [21], lead to the activation of matrix-producing cells, mainly represented by HSCs, transdifferentiating to myofibroblasts [20] (Fig. 6.2).

6.3.1 Hepatic Stellate Cells

Hepatic stellate cells (HSCs) comprise about 15% of resident cells in normal liver and approximately one-third of the non-parenchymal cells [22]. Among the different stromal cells that produce ECM, HSCs represent the main cell type implicated in the fibrogenic process, and their activation is a key event in the generation of activated myofibroblast in several conditions, including NASH [23]. HSCs are mesenchymal cells expressing desmin and glial fibrillary acid protein (GFAP), which share features with both fibroblasts and pericytes and reside in the space of Disse,

between the basolateral surface of hepatocytes and sinusoidal endothelial cells [24]. Due to this setting, HSCs exchange molecules between portal blood and hepatocytes, and can also communicate through soluble mediators with biliary epithelial cells, Kupffer cells, bone marrow-derived macrophages, infiltrating immune cells, and endothelial cells [22, 25].

HSCs are one of the major contributors to liver fibrosis [26]. In response to liver injury, HSCs undergo progressive activation and transdifferentiate from a quiescent phenotype into myofibroblast-like cells, characterized by contractile properties and production of excessive ECM components such as collagen type I and III [27, 28]. Transdifferentiation of HSCs into myofibroblasts-like cells and the maintenance of their activated state are due to autocrine and paracrine signals of several growth factors and cytokines, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF) α and β, epidermal growth factor (EGF), and connective tissue growth factor (CTGF), which induce cell proliferation, migration, ECM protein secretion, and contractility [22, 25, 29, 30].

After differentiation in myofibroblast-like cells, HSCs play a key role in ECM remodeling through the overexpression of α-smooth muscle actin (α-SMA), which drives hepatic fibrosis [31]. An imbalance between ECM synthesis and degradation is due to the activity of many mediators, such as mitogen-activated protein kinase (MAPK), integrins, and various growth factors [32]. In turn, the excessive deposition of ECM and the altered pattern of its components act as mechanical stimuli to activate HSCs [33]. During the activation process, HSCs release growth factors and cytokines, such as TGF-β, PDGF, and basic fibroblast growth factor (bFGF), to induce HSC activation in an autocrine loop to further produce ECM [34]. HSC activation can be also regulated by a plethora of chemokines, acting in different fashion, as CXCL4 which causes fibrosis, while CXCL9 has antifibrogenic properties [35]. Among these, CCL20 has recently emerged as an important proinflammatory and profibrogenic chemokines [36], and in the liver, HSCs are the main source of CCL20 [36]. Serum [37, 38] and hepatic [37] levels of CCL20 are increased in NAFLD patients with coincident fibrosis compared to those without fibrosis. Moreover, CCL20 levels increased with worsening of liver histology in NAFLD patients and during HSCs activation. In turn, CCL20 treatment of HSCs resulted in increased levels of CCL20 and ACTA2 and decreased expression of SERPIN1 and PLAU, effects mitigated by CCL20 knockdown, indicating that this chemokine has a key role in modulating ECM components released by HSCs [39]. Moreover, adipokines, secreted from adipose tissue, have been shown to influence the activated phenotype of HSCs, playing a role in the fibrogenic process [40–42].

6.3.2 Hepatocytes

Hepatocytes represent about 80% of total liver weight and about 70% of all liver cells and play an important role in the metabolic functions of this organ [43]. Excessive triglyceride storage in the liver during NAFLD takes place mainly in hepatocytes. Triglyceride accumulation is due to influx of fatty acids from the diet, de novo

lipogenesis, and FFAs released from the adipose tissue [44, 45]. The elevated levels of FFAs that reach the liver prevail on its oxidation capacity and are esterified mainly to triacylglycerol and diacylglycerol lipid fractions, which are accumulated in hepatocytes. In NAFLD patients, about 60% of hepatic fatty acids originate from adipose tissue, 25% from de novo lipogenesis, and 15% from the diet [46].

A recent study has shown that rats fed a high-fat diet show gradual, time-dependent changes in lipid content and fatty acid composition, which affects NAFLD development. There is a shift from the synthesis of beneficial fatty acids, like nervonic acid, toward the excessive accumulation of proinflammatory lipids, especially in the triacylglycerol fraction [47]. Following liver damage, injured cells release multiple products, such as damage-associated molecular patterns (DAMPs), able to contribute to the activation of HSCs and other cell types involved in the fibrogenic process. Among these, damaged hepatocytes promote fibrogenesis by releasing ROS, lipid peroxides, and apoptotic bodies [48, 49]. Hepatocytes-generated ROS may increase collagen synthesis in HSCs and induce endoplasmic reticulum (ER) stress, which in turn causes autophagy and contributes to HSC activation [50–52].

During the fibrogenic process, hepatocytes have been suggested to acquire a myofibroblastic phenotype through epithelial mesenchymal transition (EMT) [53]. Although the relevance of this pathway has been eventually challenged, one of the main pathways involved in EMT is Hedgehog (Hh) signaling which plays a key role in fibrogenesis and in tissue remodeling [54]. In NAFLD patients, Hh pathway activation is highly correlated with the severity of liver damage (e.g., portal inflammation, ballooning, and fibrosis stage) and with metabolic syndrome parameters that are known to be prognostic of advancement of liver disease. Indeed, the level of activation of the Hh pathway activity also significantly correlates with fibrosis stage [55, 56].

Hh signaling activation promotes accumulation of liver progenitors through activation of HSCs, upregulating various fibrogenic genes that lead to liver fibrosis including α-SMA, matrix molecules, matrix metalloproteinases, and tissue inhibitors of metalloproteinases, resulting in the accumulation of fibrotic extracellular matrix [57].

During this multicellular process, there is a tight interaction between liver-resident cells and infiltrating immune cells essential to the development as well as the regression of liver fibrosis and control the activation status of HSCs and hepatic ECM content. In a recent study, it has been observed that HSCs can directly induce hepatocytes to become steatotic by secreting the chemokine CCL5, revealing a novel function in the fibrogenic process [58].

6.3.3 Immune Cells

In addition to the metabolic aspects, the immune response involved in the pathogenesis of NAFLD and NASH is receiving increasing attention. The first studies that provided the evidence of the involvement of immune cells in NAFLD development were focused almost entirely on Kupffer cell activation [59, 60]. Recently,

several studies have shown that NASH is characterized by the participation of various immune subsets, both resident and infiltrating [61].

Macrophages—Intrahepatic macrophages called Kupffer cells (KCs) play an essential role in the pathogenesis of chronic liver injury [62]. Whereas in healthy liver the number of KCs remains constant, following liver damage the intrahepatic macrophages are massively expanded, due to the influx of peripheral monocytes [63]. Clinical and experimental findings have demonstrated that KC activation plays a crucial role in the initiation of liver response to an insult [64]. Indeed, KC activation mediated by FFAs, lipopolysaccharide (LPS), and endotoxin through TLR2 and TLR4 contributes to the progression of liver fibrosis by several mechanisms, including the release of metalloprotease (MMP), mainly MMP9, as well as chemokines, interleukin (IL)-1β, and growth factors like TGF-β1, VEGF, and angiotensin II that accelerate the activation of KCs. Moreover, KCs together with other liver cells (HSCs and hepatocytes) secrete chemokines, such as CCL2, promoting a massive infiltration of monocytes in the injured liver [65]. In this context, monocytes rapidly differentiate to macrophages.

Macrophages have been recently shown to display some degree of plasticity and heterogeneity [66]. Activated macrophages differentiate into two main subsets: M1 (classically activated) and M2 (alternatively activated). M1 macrophages produce proinflammatory cytokines, whereas M2 macrophages regulate inflammatory reactions and tissue repair. M2 macrophages can be further divided into diverse subtypes, each induced by different molecules and eliciting different signals. In particular, M2a macrophages are stimulated by IL-4 and IL-13, and mainly induce a Th2 response, M2b macrophages are stimulated by immune complexes and are involved in Th2 activation and immune regulation, and M2c macrophages are stimulated by IL-10 or TGF-β and are involved in immune suppression, tissue repair, and matrix remodeling [67].

Macrophage polarization is mainly regulated by signaling pathways, transcription factors, posttranscriptional regulators, and epigenetic regulation [68]. Classic regulatory pathways including JNK, PI3K/AKT, Notch, JAK/STAT, and NF-kB play a critical role in macrophage polarization [69]. In particular, the PI3K pathway can regulate many aspects of cellular functions such as metabolism, motility, and proliferation [70]. AKT is known to be an important effector of PI3K which plays a crucial role in macrophage polarization.

The balance between M1 and M2 macrophages mediates the progression or resolution of liver fibrosis. During the early stages of liver injury, bone marrow-derived monocytes are intensively recruited to the liver and differentiate into inflammatory macrophages (mostly M1) to produce proinflammatory (such as IL-1β, IL-6, IL-8, IL-12, and TNF-α) and profibrotic cytokines, promoting inflammatory responses and HSC activation. Subsequently, recruited macrophages switch to a M2 phenotype, which secrete a wide variety of MMPs (MMP2, MMP9, MMP12, MMP13, and MMP14) and anti-inflammatory cytokines (such as IL-4, IL-13, and IL-10) aimed to facilitate fibrosis resolution [71].

NKT Cells—NKT cells are the major innate-like T cells in the liver of both mice and humans [72]. Originally defined as cells expressing both the characteristic T-cell

marker CD3 and natural killer cell markers, NKT cells can be divided into two main subsets, type I or invariant NKT (iNKT) and type II, based upon differences in T-cell receptor characteristics and in the agent which they recognize [73]. Hepatic iNKT cells, following their activation, secrete cytokines which lead to HSC and KCs activation, and CD8+ T cells and neutrophil infiltration into the liver. In contrast, type II NKT cells express a relatively more diverse T-cell receptor repertoire and appear to have a regulatory role [74]. The presence of different subsets of NKT cells determines pathogenic or protective action in inflammatory liver diseases [75]. They have both fibrogenic and antifibrogenic effects. Indeed, they are characterized by release of interferon ɣ (IFN-ɣ), which leads to HSC death, while a subgroup of NKT cells shows profibrogenic properties such as the release of IL-4, IL-13, osteopontin, and Hedgehog ligands [76]. Different NKT cell subsets participate in the progression from steatosis to steatohepatitis and fibrosis in NASH. Early cytokine secreted by iNKT cells may set the initial phase during NASH progression. iNKT cells have an important role in HSC activation that mediates hepatic fibrosis following choline-deficient L-amino acid-defined (CDAA) diet that induce NASH in mice [77]. Importantly, iNKT cells secreting IFN-ɣ and IL-17A are present in high levels in peripheral blood of NASH patients [77]. The NKT cells subset plays a key role in IL-17 signaling, which has been shown to stimulate liver inflammatory cells, like KCs and macrophages, to produce proinflammatory cytokines such as IL-1, IL-6, and TNF-alpha as well as the fibrogenic cytokine, TGF-β. IL-17 also induces activation of HSCs to produce collagen type I, thus promoting their differentiation into fibrogenic myofibroblasts via Stat3 signaling pathway [78, 79].

6.3.4 Sinusoidal Endothelial Cells

Sinusoidal endothelial cells (LSECs) comprise approximately 20% of the total number of liver cells [80]. LSECs present a “dynamic, functional barrier” that preserve the liver parenchyma through an active regulation of the transfer of a wide range of solutes and macromolecules from blood to hepatocytes and vice versa [80].

Indeed, LSECs have an important role in the removal of several macromolecules from the circulation by receptor-mediated endocytosis [81]. In the healthy liver, LSECs are characterized by the presence of fenestrae and lack of a basement membrane. The lack of LSEC fenestrae directly contributes to intrahepatic resistance and hepatocellular damage [82]. Moreover, liver inflammation and fibrosis are associated with formation of basement membrane, due to deposition of extracellular matrix in the space of Disse [83].

Defenestration of LSEC contributes to various liver pathologies and plays a crucial role in NAFLD [84, 85]. LSECs play an important role in the early stages of HSC activation since fenestrated endothelial cells maintain the quiescent phenotype of HSCs [86]. In different liver diseases such as toxic, nonalcoholic, and alcoholic injuries, capillarization of endothelial cells precedes the development of fibrosis;

this process seems to be mediated by VEGF [87, 88]. Furthermore, endothelial cells produce fibronectin which converts the latent form of TGF-β in the active molecule that induces HSC activation [89].

However, it is not clear whether inflammatory activity mediated by LSEC is related with LSECs' defenestration. Recently, Kus E. et al. have shown that in high-fat diet-induced NAFLD in mice, LSECs displayed a proinflammatory phenotype that was however associated with fully preserved LSEC fenestrae and bioenergetics [90].

6.4 Lipotoxicity, Toll-like Receptors and Inflammasomes

Accumulation of FFAs in the liver drives lipotoxicity, caused by the dysregulation of the lipid environment and/or intracellular lipid composition, leading to accumulation of dangerous lipids that induce organelle dysfunction, cell injury, and death [7, 91]. Lipotoxicity may cause cellular damage by the following: (1) modifying the biology and function of intracellular organelles, such as the endoplasmic reticulum (ER) and the mitochondria; (2) modulating intracellular signaling pathways; (3) interacting with specific proinflammatory kinase receptors.

Triglycerides (TG) are physiologically stored inside the adipocytes in the postprandial period and released, when needed, through lipolysis. Small lipid droplets are present normally in hepatocytes [92], but with the ongoing expansion of adipose tissue, insulin resistance, and lipolysis, long-chain fatty acids (LCFA) are released into the circulation toward the liver, cardiomyocytes, skeletal muscle, and pancreas [93]. In these tissues, both the excess of LCFA and TG contribute to lipotoxicity, driving to clinical consequences such as NAFLD and cardiomyopathy [94, 95]. Dietary fat contributes to the hepatic uptake of LCFA and of triglycerides and serum glucose increases de novo lipogenesis [46], while insulin causes upregulation of the sterol regulatory element-binding protein 1 (SREBP1) and carbohydrate-responsive element-binding protein (ChREBP) which will further increase intrahepatic lipid amount [44]. Lipid accumulation is also promoted by the decreased LCFA β-oxidation, diminished synthesis of ApoB100 and reduced VLDL secretion [95].

Moreover, intrahepatic lipid accumulation is increased by de novo lipogenesis and adipose tissue lipolysis [96].

Several death receptors (DR5, FAS, TRAIL-R2) are upregulated by excessive influx of LCFA [97, 98] and accumulation of FFAs generate ROS, activation of caspase-2 pathway [99, 100] and JNK [101] eventually resulting in endoplasmic reticulum stress and mitochondrial impairment. Oxidative stress and cholesterol accumulation affect HSCs [102] that undergo activation with collagen production and fibrosis [103, 104].

Dying hepatocytes release extracellular DAMPs, which predispose to inflammation and fibrosis recruiting macrophages [105, 106]. Intracellular proinflammatory DAMPS include high-mobility group box 1 (HMGB1), heat shock proteins,

fibrinogen and fibronectin, and mitochondrial products such as formyl peptides and mitochondrial DNA [16]. Although they differ from pathogen-associated molecular patterns (PAMPs), bacterial products that reach the liver due to increased intestinal permeability, several DAMPS can be recognized by similar receptors, and in particular TLRs [42]. TLRs are expressed in varying levels of expression by KCs, hepatocytes, HSCs, and SECs [42]. Among NAFLD-associated TLRs, TLR2 interacts with a broad range of PAMPs, including peptidoglycan, a surface component of Gram-positive bacteria [107] which appears to be increased in NAFLD [108]. Of note, TLR2-deficient mice fed with HFD display decreased levels of inflammatory cytokines and do not develop NASH [109].

TLR9 downstream signaling involves IL-1 and is associated with NASH severity due to increased hepatic steatosis, inflammation, and fibrosis [110, 111]. TLR4 has a key role in linking innate immunity with inflammatory response. Its function is principally activated by Gram-negative bacterial lipopolysaccharides (LPS), leading to overexpression of cytokines, chemokines, and antimicrobial molecules [112, 113]. In Kupffer cells, the interaction between LPS and TLR4, which requires LPS-binding protein and two co-receptors (CD14 and myeloid differentiation protein 2), stimulates downstream pathways in a myeloid differentiation factor (MyD)88-dependent or -independent fashion [114]. MyD88-dependent pathway activation drives an increase in proinflammatory cytokines (IL-1 β, IL-6, IL-12, and TNF-α) and proteins implicated in the immune response, while the activation of MyD88-independent cascade involves IFNs [115]. In TLR4-activated Kupffer cells, ROS production also occurs, representing an additional mechanism for NAFLD progression [59]. Of note, in the presence of high glucose, TLR4 activation and downstream signaling can be activated by FFAs [116],highlighting one of the mechanisms by which saturated fatty acids have toxic effects [117].

Besides Kupffer cells, TLR4 is expressed by other liver cells, including HSCs, hepatocytes, and cholangiocytes, and the LPS/TLR4 axis plays a critical role in the pathogenesis and progression of NAFLD [59, 118]. In HSCs, TLR4 increases the expression of chemokines and adhesion molecules, as well as stimulates TGF-beta-mediated signaling [119]. Moreover, two TLR4 genetic variants, protective against fibrosis, were associated with a lower apoptotic rate of HSCs [120].

Recently it has been reported that TLR7 associates with lipid accumulation in hepatocytes by controlling lipid peroxidation and autophagy [121], and its involvement in NASH has been recently reported. In particular, it has been observed that TLR7 signaling stimulates TNF-α release in Kupffer cells and type I IFN production in dendritic cells, resulting in an increase in hepatocyte death and inhibition of the activity of Treg cells [122].

DAMPS and PAMPS lead to the assembly of inflammasome, by oligomerization domain (NOD)-like receptors (NLRs) activation. The inflammasome is a multiprotein system required for caspase-1 activity and initiation of inflammatory signals [16]. Full activation of inflammasome, mediated by pattern-recognition receptors (PRRs), via NF-kB, can be induced by different signals, such as uric acid, ATP, and ROS [123], and may lead to the development of steatosis, insulin resistance, inflammation, and cell death [124]. The ongoing mitochondrial dysfunction, oxidative

stress, and lysosomal damage occurring during NASH could also activate NLRP3 inflammasome [93]. Recent data indicate that DNA released from mitochondria, after FA stimulation, causes NLRP3 inflammasome activation, via interaction of cytosolic mtDNA with the NLRP3 inflammasome [125]. A role for inflammasomes in NAFLD development and progression to NASH has been described both in humans and in animal models [126, 127].

Recent data have also highlighted a direct role for the NLRP3 inflammasome in the activation of HSCs. Stimulated HSCs display increased levels of NLRP3 inflammasome-induced ROS production and cathepsin B activity, associated with an upregulation of mRNA and protein levels of fibrogenic markers, compared to HSCs isolated by Nlrp3$^{-/-}$ mice. Moreover, in a gain-of-function model with selective expression of mutant hyperactive NLRP3 in HSCs, upregulation of α-SMA and desmin-positive cells was observed, together with the spontaneous increase in collagen production and development of liver fibrosis. Of note, these fibrotic changes occurred without the presence of inflammatory infiltrates, supporting a direct role for NLRP3 inflammasome in the hepatic fibrogenic process [128].

6.5 Oxidative Stress, Mitochondrial Dysfunction and Hyperammonemia

Oxidative stress (OS) represents an imbalance between the production of reactive molecules and antioxidant defense [6]. The reactive molecules can contain oxygen (reactive oxygen species—ROS) or nitrogen (reactive nitrogen species—RNS) [129]. OS arises when inflammation occurs [130] and in several hepatic disorders including NAFLD. High levels of ROS and RNS are associated with the severity and progression of the disease [131]. ROS can hit vital cell constituents like proteins, nucleic acids, polyunsaturated fatty acids, or carbohydrates, and cause the disruption of the protein synthesis machinery, DNA damage, and eventually cell death [129]. ROS or RNS from mitochondria, endoplasmic reticulum, and peroxisomes plays an important role in the pathogenesis of NASH. Both ROS and RNS may inhibit mitochondrial function by posttranslational changes in the mitochondrial proteome resulting in mitochondrial dysfunction [93]. Mitochondria ROS are produced in two major respiratory chain areas: complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome C reductase) [129]. In NAFLD, FFAs overload plays a key role in ROS generation as the result of electron leakage during mitochondrial β-oxidation in ATP production. ROS production generates hepatocytes damage, inflammation, and contributes to insulin resistance [132]. As an adaptive response, FFAs induce peroxisome proliferator-activated receptors-α (PPARα) [133], a nuclear receptor that increases activity of the electron-transport chain, limiting the production of ROS [134]. However, it also inhibits the proton gradient over the inner membrane, diminishing the production of ATP, making the cell more vulnerable to ATP depletion and necrosis [135]. Furthermore, TNF-α [136, 137] and lipid peroxidation products [137] inhibit the electron-transport chain of the mitochondrion, increasing mitochondrial dysfunction and the production of

ROS [136]. Mitochondrial damage will cause in turn inhibition of lipid β-oxidation, further increasing steatosis [93, 138]. An excessive production of ROS in mitochondria can be revealed by morphological changes, including cristae swelling [139] and production of the lipid peroxidation products malondialdehyde (MDA) and hydroxynonenal (HNE) [140].

Impairments of intracellular homeostatic processes and mitochondrial function can activate both apoptotic signaling and necroptotic events [141]. Apoptosis is associated with changes in mitochondrial cardiolipin, phosphatidylcholine redox state, and increased opening of the mitochondrial permeability transition pore (MPTP), as well as release of proapoptotic proteins from mitochondrial intermembrane space [142].

Besides the release of cytochrome c and other proapoptotic factors into the cytosolic compartment, oxidative stress, lysosomal damage, and MPTP opening cause the activation of NLRP3 inflammasome and caspase 3 activation [143]. An emerging role has been recently shown for NAD+ in mitochondrial stress induction in NASH development. Mice fed high-fat/high-sucrose displayed impaired mitochondrial function associated with lower hepatic NAD+ levels [144]. Conversely, NAD+ repletion was associated with a protective effect against NAFLD, by inducing a sirtuin (SIRT)1- and SIRT3-dependent mitochondrial unfolded protein response, aimed to enhance mitochondrial activity and hepatic β-oxidation [145]. Several studies have indicated a role for coenzyme Q (CoQ), which is essential for mitochondrial respiration, in NAFLD development and progression to NASH [146–148]. Increased concentrations of CoQ have been found in the plasma and liver of NAFLD patients [149], and disturbance in CoQ metabolism was observed in experimental NAFLD during disease progression [150].

At a clinical level, enzymic and non-enzymic antioxidants have been evaluated in NAFLD/NASH. Among the non-enzymic antioxidants, bilirubin is able to scavenge reactive oxygen species (ROS) and inhibit oxidative stress [151]. Moderate increases in serum bilirubin levels were correlated with lower lipid levels and improved liver function in several patient populations [152, 153]. Moreover, mice with liver-specific knockout of biliverdin reductase A (BVRA), the enzyme that reduces biliverdin to bilirubin [154] significantly exacerbated hepatic steatosis on a high-fat diet. The loss of BVRA resulted in the reduction of the number of mitochondria, decreased expression of markers of mitochondrial biogenesis, which reduced mitochondrial oxygen consumption and increased ROS generation [155]. Additionally, inhibition of heme oxygenase (HO), an enzyme which catabolizes heme into biliverdin, ferrous iron (Fe^{2+}), and carbon monoxide (CO), resulted in increased ROS and collagen production from activated LX2, a human hepatic stellate cell line [156].

Recently findings indicate that in experimental models of NAFLD, gene and protein expression of mitochondrial urea cycle enzymes, carbamoyl phosphate synthetase (CPS1), and ornithine transcarbamylase (OTC) are diminished significantly, resulting in reduced ureagenesis and hyperammonemia [157–159]. Moreover, in non-cirrhotic NAFLD patients, a decrease in urea cycle enzymes

(UCEs) together with increased plasma and hepatic ammonia levels was observed [158, 159]. Ammonia has been shown to activate human HSCs in vitro and in vivo [160]. Conversely, reduction of ammonia concentrations prevented the activation of HSCs and decreased the severity of portal hypertension in an animal model of liver fibrosis [160]. In a rodent model of NAFLD, a progressive reduction in the expression and activity of UCEs resulting in hyperammonemia was observed, while in ammonia exposed cultured hepatocytes and precision-cut liver slices, an increase in profibrogenic marker gene expression occurred. Lowering ammonia prevented hepatocyte cell death and significantly diminished the development of fibrosis both in vitro and in vivo. These data suggest that hyperammonemia is associated with fibrogenesis development and that ammonia could be a target for the prevention of progression of fibrosis [159].

6.6 Endoplasmic Reticulum Stress and Autophagy

The endoplasmic reticulum (ER) is an intracellular organelle involved in protein assembly and its function is regulated by the unfolded protein response (UPR), whose activation leads to the degradation of misfolded proteins [161]. UPR intracellular pathways can be stimulated by proteins localized in the ER membrane, such as RNA-dependent protein kinase-like ER eukaryotic initiation factor-2α kinase (PERK), inositol-requiring enzyme-1 (IRE1), and activating transcription factor 6 (ATF6) [91]. ER stress, associated with activation of UPR, has been shown to have a key role in the pathogenesis of alcoholic or nonalcoholic fatty liver disease [162]. In NAFLD, the increased levels of diglycerides and ceramides inhibit hepatic insulin signaling pathways, contributing to the appearance of hepatic insulin resistance and ER stress [163]. Furthermore, ER stress is mediated by accumulation of diacylglyceride, phospholipid, free cholesterol (FC), and FFAs [91]. Lipids can directly induce ER stress through activation of IRE1 and PERK [164]. Saturated FFAs (such as stearic or palmitic acid), as opposed to unsaturated FFAs (such as oleic acid), can become lethal to hepatocytes, inducing ER stress and the mitochondrial pathway of apoptosis by disrupting Ca^{2+} homeostasis [165–169]. Moreover, ER stress can induce hepatocyte apoptosis through activation of C/EBP homologous protein (CHOP), a transcription factor with proapoptotic functions [170], and stimulation of the JNK pathway. Once stimulated, IRE1 can bind the adaptor protein TNF-α receptor-associated factor 2 (TRAF2) and induce apoptosis via JNK [171] or by activation of the proapoptotic proteins Bax and Bak [172].

Endoplasmic reticulum stress is also correlated with chronic inflammation, through excessive production of reactive oxygen species (ROS) and the activation of NF-κB and JNK signaling [91]. A cross talk between the ER and mitochondria has been demonstrated in cultured hepatocytes exposed to PA. This treatment provokes disruption of ER membrane and impairment of sarcoendoplasmic reticulum calcium ATPase (SERCA) activity, causing calcium efflux from ER stores and its subsequent translocation to the mitochondria, with dysregulation of mitochondrial

function and oxidative stress [173–176]. On the other hand, overexpression of SERCA levels in obese mice improves hepatic ER stress, suggesting that SERCA plays a crucial role in lipotoxic-induced ER stress and, indirectly, in mitochondrial impairment [16, 42, 176].

Of note, a cross talk between the extrinsic and intrinsic pathways of apoptosis has been observed during the progression from NAFLD to NASH [177]. In the presence of nutrient excess, macrophage-associated hepatic inflammation was found to be involved in liver injury and fibrosis by inducing TRIAL receptor signaling was found to be involved in liver injury and fibrosis [178]. Moreover, incubation of hepatocytes with PA or lysophosphatidylcholine increased the release of extracellular vesicles containing TRAIL, which induced upregulation of IL1β expression in mouse bone marrow-derived macrophages [179]. Nonetheless, the role of TRAIL in mediating the progression from NAFLD to NASH is controversial and deserves further investigation [91, 180, 181].

Induction of liver damage in different mouse models of fibrosis is associated with increased autophagy, and features of autophagy can be observed in activated HSCs, within injured human liver tissues [51]. Of note accumulating evidence indicates that ER stress in NAFLD might be linked to autophagy [182, 183]. In primary hepatocytes high glucose (HG)-induced lipid accumulation and stimulated the release of non-esterified fatty acids (NEFA) by autophagy-mediated lipophagy, and lipophagy significantly improved high glucose (HG)-induced lipid accumulation. HG-induced lipophagy activation and HG-induced changes of lipid metabolism were regulated by oxidative and endoplasmic reticulum (ER) stress pathways. Furthermore, HG-activated lipophagy and HG-induced changes of lipid metabolism acted enhancing carbohydrate response element-binding protein (ChREBP) DNA binding at the PPARγ promoter region, which in turn activated genes related to lipogenesis and autophagy. Therefore, ChREBP emerges as a key mediator of the action of glucose on lipogenic gene expression [184].

6.7 Senescence in NAFLD/NASH

Data from clinical studies suggest that NAFLD is associated with cell senescence [185]. Telomeres were found to be shorter in NAFLD patients [186], and DNA damage was higher in patients with NAFLD and increased p21 levels, indicating a cell cycle arrest in the G1/S phase [186]. Interestingly, p21 expression was associated with the fibrosis stage [186]. A possible association between telomere length and NAFLD development in T2DM patients was also investigated. Patients who developed NAFLD had shorter telomeres in peripheral blood leukocytes compared to the patients who did not have steatosis [187, 188]. In addition, genomic instability associated with cellular senescence, characterized by the presence of micronuclei, nuclear buds, and nucleoplasmic bridges, was found to be upregulated in NAFLD patients [189].

DNA methylation is another index of senescence which correlates with NAFLD and its evolution to NASH [190–193]. Hyper-methylation of the PPARγ promoter

was found in both liver tissue and circulating DNA of NAFLD patients, while PPARγ plasma DNA methylation signatures reflected the molecular pathology associated with fibrosis and thus the severity of the underlying liver disease [190]. In HSCs, dynamic changes in DNA methylation were implicated in the pivotal events of fibrogenesis, regulating the activation of HSCs into matrix-producing myofibroblasts [194].

Intriguingly, several studies have indicated that DNA methylation signatures can be used as an index of biological age [195, 196]. Peripheral blood DNA methylation signatures were used as a marker of age acceleration in NASH patients and to compare it to that of healthy controls. Patients with stage 3 fibrosis had increased aging acceleration, which was further linked to the hepatic collagen amount [197]. These data indicate that NASH may induce altered methylation features in several cells, including peripheral blood cells. Additionally, in human NASH-related hepatocarcinoma (HCC), cancer-associated fibroblasts (CAFS), and non-tumoral HSCs showed increased expression of senescence-associated secretory phenotype (SASP) markers compared to those deriving from non-NAFLD-related HCC [198].

Although the mechanisms involved in NASH resolution are not completely understood, many studies have focused on the resolution of the liver inflammation [31]. During resolution of chronic liver damage, removal of the toxic factor induces a change from an inflammatory hepatic compartment into a more restorative microenvironment, which contains circulating cells that lead to degradation and collapse of excessive extracellular matrix, secreting fibrolytic MMPs and by inducing senescence or apoptosis of activated HSCs [31, 199]. Senescent HSCs produce less extracellular matrix components and more MMPs, thereby contributing to fibrosis regression [200]. These data suggest that hepatic senescence, induced by HFD and aging, characterizes the pathogenesis of NAFLD, but at the same time may also have a beneficial impact on the progression from NAFLD to NASH [185].

6.8 Genetic Factors Implicated in Fibrogenesis of NAFLD

Different genetic factors can contribute to the development of liver steatosis and fibrosis, as different individuals with similar risk factors can develop NAFLD and NASH at different stages. Genome-wide association studies (GWAS) have become a novel tool in assessing which genes are potentially associated with a particular disease and, within it, with a particular phenotype [201–203]. The first report of a relationship of NAFLD with a single nucleotide polymorphism (SNP), identified the patatin-like phospholipase domain-containing 3 (PNPLA3) on chromosome 22, PNPLA3 rs738409, as a prime candidate for genetic-associated NAFLD [204]. This variant form corresponds to the amino acid substitution [I]>[M] at position 148, and correlates with higher liver lipid content and predisposes to fatty liver-associated liver disease, from simple steatosis to steatohepatitis, fibrosis, and HCC [205]. Overexpression of the I148M variant in mouse liver induced accumulation of triacylglycerol, increased synthesis of fatty acids, and reduced hydrolysis of triacylglycerol [206]. To date, three large-scale meta-analyses [207–209] have been

performed across a vast array of ethnicities and large sample populations indicating that PNPLA3 rs738409 is the major genetic variant implicated in the pathogenesis of NAFLD [210]. Recently HSC and hepatocytes have been shown to express PNPLA3, which may regulate retinol metabolism and cell biology, as retinol serum levels and hepatic content of retinol are influenced by the PNPLA3 genotype [211, 212]. Of note, the PNPLA3 gene and protein expression increase during the early phases of transdifferentiation and remain elevated in fully activated HSCs. Furthermore, the HSCs from I148M donors show higher expression and release of proinflammatory cytokines, such as CCL5, GM-CSF, and TGF-β, thus contributing to migration of immune cells [213]. I148M HSCs showed also reduced LXRα levels and signaling compared to WT, leading to cholesterol accumulation. The use of a specific LXR agonist displayed beneficial effects inhibiting sustained HSC activation and development of liver fibrogenesis. These results indicate that the PNPLA3 genotype contributes to differential recruitment of inflammatory cells and impaired cholesterol and lipid metabolism in HSCs, regulating the severity of hepatic fibrosis [214].

Like PNPLA3, HSD17B13 is also located in lipid droplets [215] and regulates synthesis of lipid and modulates the activation of steroid hormone receptors in target tissues [216]. HSD17B13 is primarily expressed in the liver, and it is highly expressed in NAFLD patients [217]. The association of genetic variants of HSD17B13 with progression of NAFLD is complex, with different SNPs associated with different phenotypic patterns.

Some variants in HSD17B13 were associated with histological degree of steatosis, including rs6834314 and its associated splice SNP rs72613567, and other independent SNPs. Recently, the association of rs6834314 in HSD17B13 with ALT has been observed, suggesting a key role of HSD17B13 in liver injury [218]. Ma et al. have observed a variant in HSD17B13 (rs62305723) that abolishes retinol dehydrogenase activity in vitro and is associated with reduced inflammation and ballooning in a large cohort of patients [218]. Another variant in (HSD17B13, rs72613567:TA) encoding for a splice variant was associated with protection against both alcoholic and nonalcoholic progressive fatty liver disease [219].

Different studies have validated the association of the genetic variant rs58542926 in transmembrane 6 superfamily member 2 (TM6SF2), located on chromosome 19 (19p13.11), with NAFLD [220–222]. The variant rs58542926 in TM6SF2 modulates hepatic fibrogenesis [222, 223] and correlates with histological severity of steatosis, hepatic inflammation, and fibrosis [224]. This mutation is associated with steatosis and the increased risk of advanced fibrosis, independently of other factors, including obesity, diabetes, or PNPLA3 genotype. TM6SF2 is a lipid transporter [225], and the amino acid change E167K causes its loss of function with the consequent reduction of lipoproteins and apolipoprotein B (APOB) levels, resulting in an increase in hepatic deposition of triglycerides and the amount and size of lipid droplets. Conversely, the size and number of lipid droplets decreased when TM6SF2 was overexpressed, suggesting that TM6SF2 plays a role in regulating liver lipid efflux [221, 225]. Kozlitina et al. have observed a correlation between the *TM6SF2* E167K variant and the increased serum ALT and AST levels

through relevant studies in three cohorts. Moreover, Dongiovanni et al. have investigated 1201 patients with suspected NASH who were diagnosed by liver biopsy and reported that the E167K variant may increase the risk of NASH and advanced fibrosis. The mechanism seems correlated to the reduction of very low-density lipoprotein (VLDL) secretion, resulting in TG accumulation and consequent steatosis [224]. These data suggest that the variant may determine the severity of the disease, leading to a more severe degree of steatosis and a greater risk of developing steatohepatitis.

Membrane-bound O-acyltransferase domain-containing 7 (MBOAT7) (also known as lysophospholipid acyltransferase) catalyzes acyl chain remodeling of phosphatidylinositols, binding arachidonic acid to lysophosphatidyl inositol and decreasing free arachidonic acid levels [226]. Arachidonic acid causes hepatocyte apoptosis, triggering hepatic inflammation and fibrosis [227, 228]. The rs641738 C>T genetic variant of MBOAT7 was identified by GWAS in alcoholic liver disease in which it augments the risk of cirrhosis [229]. Moreover, it is involved in hepatitis B and C, and in conferring a risk of HCC in non-cirrhotics with NAFLD [230–233].

The rs4374383 AA genotype of the receptor tyrosine kinase Mer (MERTK) correlated to lower MERTK hepatic expression and resulted protective against F2–F4 fibrosis in patients with NAFLD. MERTK is a receptor of the TAM family, with a crucial role for the initiation of efferocytosis, a process by which dying cells are removed by phagocytes [233, 234]. MERTK was found to be overexpressed in activated mouse HSCs and in an experimental model of liver fibrosis [235]. A polymorphism for MERTK has been very recently associated with advanced fibrosis in NAFLD [234]. Interestingly, in human NAFLD specimens, MerTK was mainly expressed in macrophages and HSCs, and MERTK promoted HSCs activation, thus resulting in excessive fibrogenesis by abundant collagen and extracellular matrix protein secretion [234]. In a recent study, the MERTK rs4374383 G>A variant protected against 9-year incident NAFLD and diabetes, while MERTK A-allele carriers had higher fat oxidation rates and tissue insulin sensitivity [236].

Kruppel-like factor 6 (KLF6) belongs to the Kruppel-like family of transcription factors known to have different roles in differentiation, development, cell growth, apoptosis, and angiogenesis. It was identified as an early factor expressed in activated hepatic HSCs after liver injury [237, 238] raising the possibility of its involvement in the fibrogenic process, regulating collagen 1, transforming growth factor-β1, and types I and II transforming growth factor-β receptors [238, 239]. Moreover, KLF6 increased in response to oxidative stress in a model of nonalcoholic steatohepatitis, providing support for a role of KLF6 in NAFLD [240]. The rs3750861 variant of KLF6 was found to inhibit activation of HSCs after liver injury, thus reducing fibrosis [241].

Hepatic iron deposition induces fibrogenesis through multiple pathways including oxidative stress and direct activation of HSCs [242, 243]. Genetic variants in the HFE, beta-globin, and TMPRSS6 genes influence hepatic iron accumulation and are associated with hepatic fibrosis in NAFLD patients [244, 245]. Several genetic polymorphisms associated with cell senescence have also been correlated with fibrosis in NAFLD. In fact, hepatocyte senescence leads to hepatic fibrosis by

the activation and proliferation of HSC [186]. Loss-of-function mutations in the telomerase reverse transcriptase (TERT) gene, which regulates DNA damage and cell senescence, have been associated with familial liver disease and accelerated development of cirrhosis and HCC in NAFLD and other chronic liver disease [246, 247]. Interestingly the genetic variant rs762623 SNP in the CDKN1A gene, which encodes p21, has been linked to fibrosis in a cohort of NAFLD patients [248].

6.9 Adipokines and Myokines

In obesity, the adipose tissue enlarges due to an enhanced storage of lipids in white adipocytes, but the impaired lipid storage capacity of this tissue contributes to increased lipid deposition in non-adipose tissues, such as the liver. Obesity modifies the secretion of proteins, called adipokines, from adipose tissue, and alterations in adipokine secretion have been associated with the inhibition of insulin effect in peripheral tissues like the liver and skeletal muscle, and also with the development of diabetes-associated diseases including NAFLD [249].

Among the adipokines produced by the adipose tissue, leptin plays a major role in energy metabolism. Following an increase in adipose tissue mass, leptin is upregulated, acting as compensatory factor in maintaining insulin sensitivity and exerting anti-steatotic effects. Nevertheless, if adipose tissue continues to enhance, the compensatory mechanism fails, with a sustained rise in insulin resistance and hepatic steatosis [250]. Leptin-mediated dual action has been shown in experimental NAFLD. In early disease, leptin exerts a protective effect by inhibiting hepatic glucose production and de novo lipogenesis through induction of fatty acid oxidation, while, as NAFLD evolves, it acts as a pro-fibrogenic and inflammatory factor [16].

Similarly to proinflammatory cytokines, leptin is involved in differentiation of Th1 cells in adipose tissue, CD8+ T cells, mast cells, and macrophages and stimulates the expression of proinflammatory cytokines such as TNF-α, IL-6, and IL-12 [251]. Decreased concentrations of leptin in plasma indicates malnutrition and leads to impairment of immune system function [252]. In addition, leptin participates in NASH also through regulating HSCs [253]. In fact, leptin reduces peroxisome proliferator-activated receptor γ (PPARγ) that exerts a key role in the inhibition of HSC activation, thus promoting liver fibrosis [254]. Recent data suggest that leptin inhibits SREBP1c expression, which plays a crucial role in inhibiting HSC activation, through upregulation of miR-27a/b-3p levels [41, 255].

Adiponectin is one of the most abundant adipokines, and is also released by hepatocytes in response to liver injury [256]. Adiponectin levels drop when adipose mass increases, and decreased levels of adiponectin correlates with metabolic syndrome and NAFLD. Adiponectin decreases the levels of proinflammatory cytokines (including TNF-α) and induces anti-inflammatory cytokines (IL-10), leading

to impaired macrophage function [257]. Adiponectin also alleviates oxidative stress and fibrogenesis acting on HSCs, Kupffer, and sinusoidal cells [258]. Recent data suggest a possible mechanism for the inhibitory effect of adiponectin on HSC activation. Adiponectin induced upregulation of miR-29b in HSCs, which can suppress DNMT3B, thus resulting in inhibited methylation of PTEN CpG islands ultimately suppressing the PI3K/AKT pathway [259].

Another adipokine, adipocyte fatty acid-binding protein (AFABP), is a member of the lipid chaperone fatty acid–binding protein family and is produced by adipocytes, macrophages, as well as Kupffer cells [260]. AFABP levels, which correlates with subcutaneous, but not visceral fat, independently predicted inflammation and fibrosis in NAFLD [261]. Adipose tissue also produces irisin encoded by the fibronectin type III domain-containing protein 5 (FNDC5) gene [262, 263]. Irisin has been linked to increased thermogenesis and reduction of insulin resistance by stimulating glycogenesis and by reducing gluconeogenesis in animal models [262–265]. In humans, circulating levels of irisin have been associated with a wide spectrum of metabolic diseases, varying from obesity and insulin resistance to diabetes [266, 267] and cardiovascular diseases [268]. In a study conducted in NAFLD and NASH patients, similar circulating levels of irisin were observed in the two groups, and irisin was independently and positively associated with the presence of portal inflammation and fibrosis [269]. In following studies, irisin levels were found to be increased with the grade of steatosis and the stage of fibrosis [270, 271]. Irisin was found to be expressed in human activated HSCs, where it mediated fibrogenic actions and collagen synthesis, suggesting an important role in regulating liver fibrosis. Furthermore, the FNDC5 rs3480 variant was correlated with protection from clinically significant fibrosis in patients with NAFLD [271]. Finally, the 148 M allele of the PNPLA3 gene was found to be correlated with plasma irisin levels in a children cohort, indicating that this myokine is strictly connected to the development of NAFLD [272].

Myostatin is a negative modulator of muscle growth and trophism [273] and acts via interaction with activin receptor-2B (ActR2B) [274]. Elevated expression of myostatin has been reported in the muscle of patients with type 2 diabetes, and myostatin levels are reduced in the skeletal muscle of obese individuals undergoing weight reduction, highlighting the possibility that the muscle–liver axis plays a relevant role in the pathogenesis of NAFLD [275, 276]. Recent findings indicate that ActR2B is upregulated in the liver of mice with experimental fibrosis, and is detectable in HSCs. Moreover, HSCs contribute to ActR2B expression also in liver tissue from patients with NASH and fibrosis, although other cells were also positively immunostained for this protein. Myostatin inhibited HSCs proliferation, induced cell migration, and increased expression of procollagen type 1, TGF-β, and tissue inhibitor of metalloproteinase-1 (TIMP-1) through activation of the JNK signaling pathway. These data suggest a novel muscle-to-liver pathway potentially implicated in the pathogenesis of hepatic fibrosis in NAFLD [277].

6.10 Concluding Remarks

As outlined in this chapter, the process of fibrogenesis is very complex and is regulated by cues arising within the liver and from extrahepatic tissues, including the gut, and the adipose tissue. Fibrosis is the major feature of NASH related to liver-related and all-cause mortality. All trials directed to patients with NASH currently enroll subjects with significant or severe fibrosis. Therefore, understanding the mechanisms leading to the development of the fibrogenic process is critical to develop new therapeutic strategies and to possibly identify biomarkers.

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The Natural History of NAFLD: Environmental vs. Genetic Risk Factors

7

Luca Valenti and Serena Pelusi

7.1 Features of NAFLD

Nonalcoholic fatty liver disease (NAFLD) is now the leading cause of liver disease worldwide [1], and prevalence is still on the rise [2]. The hallmark of NAFLD is represented by hepatic fat accumulation exceeding 5% of liver weight, which is not explained by at- risk alcohol intake, usually defined by a threshold of 30/20 g/day in males/females. The main risk factors for the disease are represented by obesity, the constellation of metabolic alterations associated with insulin resistance (the so called metabolic syndrome) and type 2 diabetes. In most of the cases, NAFLD represents the hepatic manifestation of insulin resistance [3]. Hepatic fat is mainly accumulated within intracellular lipid droplets in hepatocytes, under the form of triglycerides. Indeed, the esterification within triglycerides represents the safest way to store free fatty acids, which derive from the adipose tissue due to systemic insulin resistance and from *de novo* lipogenesis stimulated by hyperinsulinemia, and would otherwise cause severe lipotoxicity and activation of fibrogenesis [4]. However, lipid droplets formation, metabolism and catabolism are highly regulated, and several proteins involved in the pathogenesis of liver damage and potentially lipotoxic compounds are involved in this biological process [5].

The acronym NAFLD defines a wide spectrum of liver conditions, ranging from simple uncomplicated steatosis to forms of liver disease associated with hepatocellular damage ("ballooning") and lobular inflammation, which is non-alcoholic steatohepatitis (NASH) [6]. NASH is more commonly associated with activation of

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hepatic fibrogenesis, initially at pericellular and perivenular level, which in susceptible individuals may lead to cirrhosis and advanced liver disease. The pathogenesis of the transition from simple steatosis to NASH or progressive disease is still not completely understood, and likely multifactorial [7]. Altered microbiota and gut permeability, the severity of metabolic alterations, oxidative stress and a proinflammatory imbalance in the release of mediators from the adipose tissue and the muscle are likely involved.

7.2 Natural History of NAFLD

The knowledge on the natural history of liver disease related to NAFLD is still limited by the relatively low number and the selection bias of patients with histological characterization of liver damage with available long-term follow-up, and conversely by the lack of detailed characterization of liver damage for most individuals included in prospective population studies. However, a few robust conclusions could be established. The first one is that a diagnosis of NAFLD seems to be associated with an increased mortality rate, the leading cause being cardiovascular disease, followed up by extra-hepatic cancer and liver disease (the latter with the higher relative risk as compared to the general population) [8–11]. Heightened cardiovascular risk seems to be related to accelerated atherogenesis, independently of classic risk factors [12, 13], but may also reflect more severe insulin resistance with increased susceptibility to develop type 2 diabetes [14].

Secondly, the main prognostic determinant in patients with NAFLD is represented by the severity of liver fibrosis [15]. Overall evidence indicates that, compared to NAFLD patients with no fibrosis, NAFLD patients with fibrosis are at an increased risk for all-cause mortality, and this risk increased with increases in the stage of fibrosis. When NAFLD-related fibrosis was estimated non-invasively, this conclusion held true also in the general population, and the association was independent of several possible confounding factors [16]. Most importantly, the impact of fibrosis is more pronounced for liver-related mortality as the risk of liver-related mortality increased exponentially with each increase in the stage of fibrosis, even if these estimates could not be corrected for age [15]. For stage 1, mortality rate ratio was estimated at 1.41 (95% confidence interval (CI) 0.17–11.95); stage 2, 9.57 (95% CI 1.67–54.93); stage 3, 16.69 (95% CI 2.92–95.36); and stage 4 (cirrhosis), 42.30 (95% CI 3.51–510.34) [15]. In particular, in patients with cirrhosis, liver disease becomes the leading cause of death [17, 18]. Conversely, cardiovascular disease and extra-hepatic cancer predominate in those with lower fibrosis stages, but their incidence as liver function begins to deteriorate [17]. In contrast, although the presence of NASH overall is also associated with increased mortality as compared to simple mild steatosis [19], NASH without liver fibrosis does not seem to confer an increased risk of mortality [20, 21]. Indeed, fibrosis progression rate is influenced by basal fibrosis stage. Although there is a wide variability in the transition rates between different stages of fibrosis, estimates are consistent with lower rate transition between no to mild fibrosis (0.3–2.2%) than between intermediate to

advanced fibrosis (2.8–13.3%) [2]. It should be taken into account that these estimates also account for disease regressors, about one-third of patients in prospective studies, mostly represented by individuals who lose weight or improved metabolic control during the follow-up [22].

However, the presence of histological NASH is likely associated with faster progression, on average, of liver fibrosis [23–25], in keeping with a role of oxidative stress and immune activation in determining the evolution of this liver disease. In particular, a meta-analysis of early studies led to an estimation that to progress of one fibrosis stage, it takes on average 14 years in patients with steatosis, while only 7 years in those with NASH, but fibrosis progression occurs in only about one-third of patients. However, there is a wide variability, and a significant proportion of patients without histological NASH show rapid progression of liver disease. Furthermore, even in patients without NASH, the presence of mild histological inflammation may be associated with a higher risk of disease progression [26].

The increasing prevalence of this condition, and especially due to the ageing of affected individuals and more and more frequent association with type 2 diabetes, is leading to a dramatic rise in the proportion of patients who may develop advanced fibrosis [2]. Indeed, NAFLD is already becoming a leading indication for liver transplantation in Western countries [27]. Hepatocellular carcinoma is also a rising cause of liver disease included in the disease spectrum of NAFLD [28, 29]. Although the risk of progression is higher with increasing severity of liver fibrosis, it should be noted that HCC may also develop in patients with NAFLD with significant liver disease, rendering surveillance, early diagnosis and application of curative treatments difficult tasks [30, 31].

7.3 Environmental Risk Factors for Disease Progression

The severity of the metabolic abnormalities, insulin resistance, and in particular the presence of type 2 diabetes represent the major risk factor associated with development of advanced liver disease, and with fibrosis progression in prospective studies in patients with NAFLD [23–25]. A key mediator of liver disease progression induced by metabolic risk factors may be represented by severity of hepatic fat accumulation, which has been linked with short- and long-term fibrosis progression independently of several confounders [32–34]. Cohort studies also highlighted a possible role of arterial hypertension as a risk factor for progressive worsening of fibrosis, possibly due to activation of the neurohormonal sympathetic system leading to stimulation of hepatic stellate cells. In keeping, variations in body weight and associated metabolic abnormalities represent the main clinical predictor of the liver disease evolution during the follow-up.

Cross-sectional studies also highlighted that independently of adiposity, physical exercise may have a protective role, while sarcopenia is associated with more severe liver damage. Furthermore, besides total caloric intake, the quality of diet also matters. Indeed, industrial fructose intake has been associated with higher risk of both development and progression of NAFLD, probably by inducing ATP depletion,

stimulating lipogenesis and decreasing lipid oxidation [35], and an increase in the ratio of dietary saturated/unsaturated fat intake may also play a role. On the other hand, the role of red meat consumption is less established. Concerning beverages, besides a predisposing role of sodas containing fructose, a moderate intake of alcohol with wine, but not with beer, and not under the form of binge drinking, was associated with protection from fibrosis in cross-sectional epidemiological studies, but any alcohol intake was associated with increased risk of disease progression in those with clinically significant fibrosis [36]. Vice versa, coffee consumption may be protective by promoting the antioxidant response.

Lastly, some drugs active on cardiovascular risk factors may influence the risk of liver damage progression, e.g. statins and renin angiotensin aldosterone axis modulators may reduce fibrogenesis by reducing free cholesterol and altering activation of hepatic stellate cells. Exposure to environmental toxins may also play a role in disease susceptibility, but few data are available in the literature.

A cartoon depicting the natural history of liver disease in NAFLD is presented in Fig. 7.1. Given the wide uncertainty concerning the progression (and possible regression) rates across the different stages of the disease, which may vary according to the specific populations, wide confidence intervals are indicated.

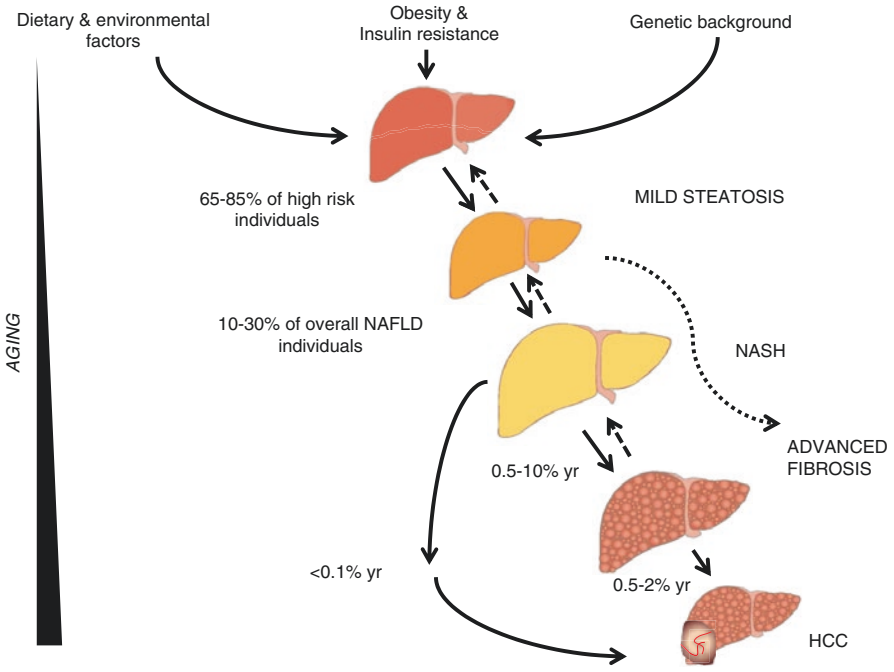

Fig. 7.1 Natural history of NAFLD. The majority of individuals with environmental, metabolic and genetic risk factors develop some form of NAFLD, and 10–30% of them NASH. With time and ageing, NASH can progress to advanced fibrosis at variable rates (although progression from simple steatosis cannot be excluded) and then to liver failure or hepatocellular carcinoma (HCC). *Yr* per year

7.4 Role of Heritable Factors in NAFLD

Accumulating evidence indicate that hepatic fat and NAFLD are strongly heritable conditions [37]. First, twin studies led to the estimation that in the general population more than half of the variability of aminotransferases levels in individuals without viral hepatitis or alcohol abuse and of hepatic fat content are accounted for by heritable factors [38, 39]. Interestingly, they also suggested that heritability of liver fat and fibrosis share are shared traits, in line with a causal role of hepatic fat accumulation in triggering progressive liver disease [38, 39]. Second, multi-ethnic cohort studies demonstrated that there is a strong interethnic variability in the susceptibility towards NAFLD development, being higher in Hispanics, intermediate in Europeans and lower in African Americans, independently of adiposity, type 2 diabetes and socioeconomic factors [40]. Lastly, family studies showed that cases of NAFLD progressing to advanced fibrosis tend to cluster in specific families. Indeed, the risk of progressive NAFLD is higher in first-degree relatives of patients with NAFLD cirrhosis as compared to the general population, independently of several confounders [41].

7.5 Genetic Determinants of NAFLD Development and Progression: The PNPLA3 I148M Variant

The most important common genetic determinants of hepatic fat variability and the susceptibility to develop NAFLD have been uncovered thanks to the advent of genome-wide association studies in the last years. The major one is the rs738409 C>G encoding for the I148M protein variant of Patatin-like phospholipase domain-containing 3 (*PNPLA3*), accounting for a large fraction of the increased risk of this condition in Hispanics [42]. The I148M variant is specifically associated with increased hepatic fat, without major influences on adiposity, circulating lipids and risk of type 2 diabetes [42]. Importantly, the I148M variant increases susceptibility to the whole spectrum of liver damage related to NAFLD, from simple steatosis to NASH, fibrosis and cirrhosis, thereby representing a general modifier of liver disease progression [43]. Furthermore, the I148M variant heightens the risk of progression to hepatocellular carcinoma development independently of the effect on fibrosis. In Europeans, homozygosity for the mutation is enriched almost ninefold in patients who develop NAFLD-HCC as compared to the general population, while absence of the variant can rule out HCC risk with a high specificity in the general population [43–45]. Homozygosity of the mutation is also associated with increased risk of hepatic decompensation in patients with fatty liver and portal hypertension [46].

Carriage of this variant impacts on the risk of liver disease particularly during the developmental age [47, 48], interacting with dietary factors such as intake of fructose-enriched drinks and lack of physical activity [49]. However, the major environmental trigger of the phenotypic expression of the variant in those who

do not drink excess alcohol is represented by excess adiposity [50]. Indeed, during obesity and insulin resistance, the PNPLA3 protein is induced by insulin and expressed at the surface of lipid droplets, where it mediates the remodelling of triglycerides and phospholipids, in particular by mediating the hydrolysis of oleic acid [51]. The mechanism behind the association with steatosis is related to accumulation of the mutated I148M protein on the surface of lipid droplets altering lipid remodelling and turnover [51–53]. Furthermore, the I148M alters retinol release from hepatic stellate cells directly favouring inflammation and fibrogenesis [54–56].

7.6 Other Common Genetic Determinants of NAFLD

Other common genetic mutations regulating hepatocellular lipid handling contribute to the risk of NAFLD. The rs58542926 C>T encoding for the E167K variant in Transmembrane 6 superfamily member 2 (*TM6SF2*) favours hepatic fat accumulation by decreasing lipid secretion in very-low-density lipoproteins (VLDL), also leading to increased susceptibility to liver damage. At the same time, this genetic factor protects from cardiovascular disease by reducing circulating lipids [57–59]. Variants in *Glucokinase regulator* (*GCKR*) [60, 61] and in membrane bound O-acyl transferase 7 (*MBOAT7*) [62] also contribute to the risk, by increasing de novo lipogenesis and altering the remodelling of phospholipids, respectively. All these factors result in fat accumulation and higher risk of liver disease. Conversely, a variant in protein phosphatase 1 regulatory subunit 3B (*PPP1R3B*) has recently been demonstrated to protect against hepatic fat accumulation, possibly by shunting the excessive energy supply towards glycogen synthesis [63]. This also resulted in decreased risk of progressive liver disease in individuals at high risk of NASH. All in all, a general concept emerging from these studies is that the risk of progressive NAFLD is strongly related and proportional to the impact of these genetic risk factors on hepatic fat accumulation, suggesting this is a major driver of liver damage progression [33].

However, other genetic variants may modify the effect of fat accumulation on inflammation and fibrosis. The best studied are represented by variants regulating oxidative stress and innate immunity in the liver. Indeed, variants in the mitochondrial Mn-superoxidase dismutase 2 (*SOD2*), and uncoupling protein 2 (*UCP2*), regulating fatty acid oxidation and redox status in the mitochondria [64, 65]. Concerning inflammation, variants in the interleukin 28 (*IL28*) locus encoding for the alternative interferon lambda-3 and lambda-4 proteins, and those in Mer T kinase (*MERTK*), regulating the activation of phagocytes and hepatic stellate cells [66]. Very recently, a common variant in 17-beta hydroxysteroid dehydrogenase 13 (*HSD17B13*) encoding form of this enzyme that is expressed at the surface of lipid droplets in hepatocytes has been identified. This polymorphism protects against progressive liver disease associated with fat accumulation particularly in carriers of the I148M *PNPLA3* variant [67, 68].

7.7 The Role of Rare Inherited Mutations

Noteworthy, rare genetic mutations with a strong impact on the function of proteins involved in NAFLD pathogenesis may also contribute to determine the predisposition to develop advanced NAFLD and disease clustering in specific families. For example, mutations in Apolipoprotein B (*APOB*) favour disease progression again by causing lipid compartmentalization in hepatocytes [69]. This is caused by the inability to secrete VLDL from hepatocytes. However, as APOB is also involved in the secretion of chylomicrons, damage to the intestinal barrier due to accumulation of lipids in enterocytes, and malabsorption of liposoluble vitamins (and especially A, D, E may be relevant for the risk of NAFLD) may also occur. Another mechanism leading to progressive NAFLD is related to telomere shortening and cell senescence [70], usually mediating the effect of ageing on the risk of liver disease, and mutations in *telomerase reverse transcriptase* (*TERT*) have been associated with progressive NAFLD [71, 72]. Finally, it should not be forgotten that in children NAFLD may represent the manifestation of severe genetic disorders, such as lysosomal acid lipase deficiency caused by mutation of *LIPA* gene, which determine accumulation of cholesteryl esters and triglycerides in hepatocytes [73].

An overall picture of genetic loci associated with NAFLD development and progression, classified according to the role on encoded proteins in the accumulation of lipids (lipogenesis, lipid oxidation, lipid droplets formation and remodelling, lipid secretion within VLDL) and development of liver damage (lipotoxicity, inflammation and activation of fibrogenesis) is shown in Fig. 7.2.

7.8 The Role of Epigenetic Changes

The term "epigenetic changes" refer to relatively stable alterations of nuclear DNA and the mechanisms of transcriptional regulation that can be transmitted through cell division. These are involved in mediating the effect of environmental factors on phenotype, and may possibly explain part of the missing heritability and variability of disease progression of common diseases such as NAFLD. Methylation of cytosine nucleotides at CpG-rich regulatory or promoter regions represents the first level of regulation of gene expression. Several post-translational modifications of histones also contribute to modulating the access of transcription and regulatory factors to the DNA. An important role of epigenetic factors in modulating the susceptibility to NAFLD is demonstrated by the effect of intrauterine exposure to high-fat diet in experimental models, leading to more severe hepatic fat accumulation and the development of NASH [74]. These experiments recapitulate the effect of an adverse foetal environment on the risk of NAFLD. Indeed, both intrauterine growth retardation and accelerated foetal growth are associated with an increased risk of NAFLD and NASH [75–78]. In keeping, hepatic DNA tends to be demethylated in patients with NAFLD [79]. Genes involved in the methylation process, lipid metabolism (including *PNPLA3*), inflammation and fibrogenesis showed stage-dependent

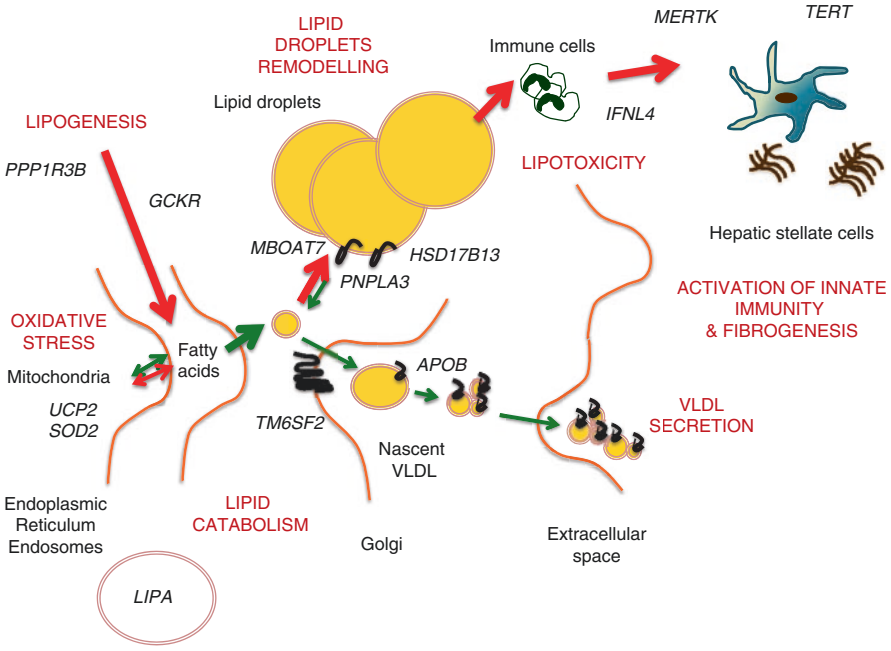

Fig. 7.2 Genetic loci involved in the development and progression of NAFLD, classified according to the mechanism by which the encoded proteins intervene in the pathogenesis of the disease. Red arrows indicate pathological processes/lipid fluxes, green arrows beneficial pathways. *PPP1R3B* Protein phosphatase 1 regulatory subunit 3B, *GCKR* glucokinase regulator, *UCP2* uncoupling protein 2, *SOD2* mitochondrial superoxide dismutase, *LIPA* lysosomal acid lipase, *PNPLA3* patatin-like phospholipase domain-containing 3, *MBOAT7* membrane bound O-acyl transferase 7, *HSD17B13* 17-beta hydroxysteroid dehydrogenase 13, *TM6SF2* transmembrane 6 superfamily member 2, *APOB* apolipoprotein B, *VLDL* very-low-density lipoproteins, *IFNL4* interferon lambda 4, *MERTK* Mer T kinase, *TERT* human telomerase reverse transcriptase

regulation, suggesting that epigenetic changes are involved in the progression of liver disease [79, 80]. These alterations confer an especially high risk of liver disease in patients born with a strong genetic predisposition.

Another layer of regulation is provided by non-coding RNAs. Indeed, NAFLD is associated with deregulation of many hepatic micro-RNAs (miRNA) [81, 82]. The most robustly validated alteration is represented by downregulation of miR-122, [81–85], which promotes lipogenesis [81], and in experimental model is associated with spontaneous development of NASH and HCC [83]. However, several miRNAs are altered during NASH, and their variability seem be involved in mediating the susceptibility to the disease [37, 86].

7.9 Interaction Between Genetic and Environmental Factors

The phenotypic expression of the disease is triggered by the interaction between the genetic background and environmental triggers. The most common one is represented by increased adiposity, leading to insulin resistance and hyperinsulinemia

[87]. For example, at the general population level most of the carriers of *PNPLA3*, *TM6SF2* and *GCKR* common risk variants are not affected by NAFLD, and most importantly do not develop progressive liver disease. However, the impact of the variants on hepatic fat content, the risk of NAFLD and that of cirrhosis increases exponentially with increasing BMI, indicating the presence of a synergism between these components of the disease [88]. Similarly, there seems to be an interaction between consumption of industrial fructose in soft drinks and the *PNPLA3* I148M variant in determining the susceptibility to NAFLD [49]. On the other hand, omega-3 fatty acids would be less effective in reducing lipogenesis and liver fat in carriers of this variant [89, 90]. Importantly, in individuals at high genetic risk a healthy dietary patter modelled on the Mediterranean diet may reduce the risk of NAFLD [91], as well as regular physical activity may prevent disease development [49].

An overview of common inherited and acquired factors involved in the development and progression of NAFLD is presented in Fig. 7.3 [78].

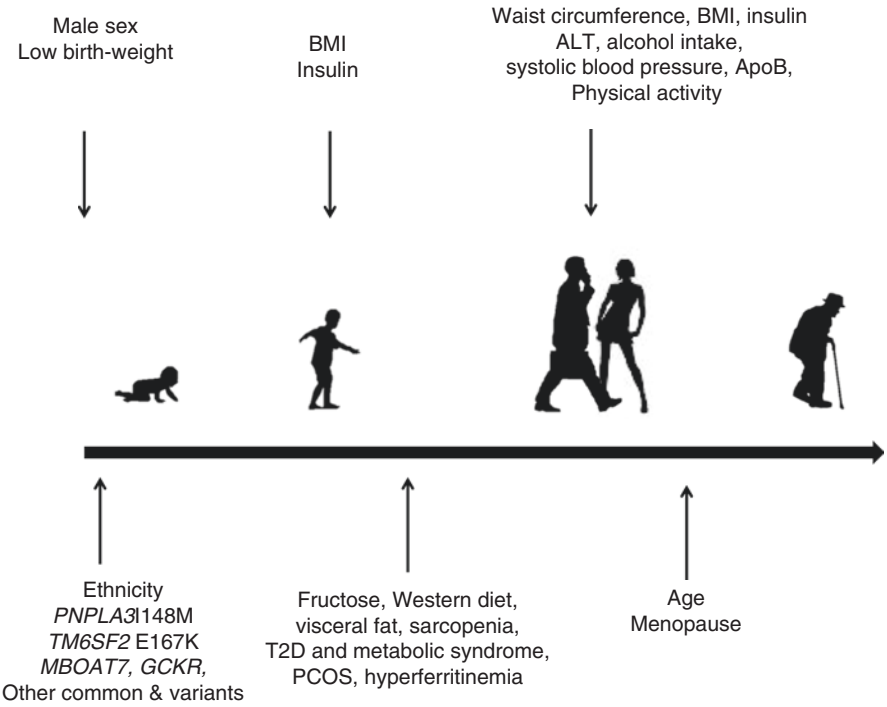

Fig. 7.3 Complementary role of inherited and acquired risk factors for NAFLD according to life stages. *BMI* body mass index, *ALT* alanine aminotransferases, *T2D* type 2 diabetes, *PCOS* polycystic ovary syndrome, *PNPLA3* patatin-like phospholipase domain-containing 3, *MBOAT7* membrane bound O-acyl transferase 7, *GCKR* glucokinase regulator, *TM6SF2* transmembrane 6 superfamily member 2

7.10 Possible Future Clinical Applications of Genetics

Variants in *PNPLA3* and *TM6SF2* are strong risk factors for NAFLD, especially in individuals with strong predisposition, such as obese adolescents with severe insulin resistance developed after intrauterine growth retardation. Genotypization of these common variants is able to significantly improve the prediction of the risk of severe progressive NAFLD, hopefully allowing to tailor preventive lifestyle approaches in the future [78]. Furthermore, the number of common genetic risk variants for hepatic fat accumulation in *PNPLA3*, *TM6SF2*, and *MBOAT7* nicely stratify the risk of NAFLD in the general population, interacting with adiposity [50, 62]. Notably, the same simple genetic instrument is able to predict the risk of HCC in patients with NAFLD independently of classic risk factors, possibly improving risk stratification for this condition, even in patients without severe fibrosis [92]. An emerging concept is that genetic risk variants for progressive liver disease related to NAFLD may protect at the same time from dyslipidemia and cardiovascular disease. This is particularly true for those that have inhibition of lipid secretion within VLDL as the main mechanism, such as those in *TM6SF2* and *APOB*, and also the *PNPLA3* I148M mutation. Therefore, they may be useful to dissociate the risk of hepatic vs. cardiovascular complications of insulin resistance, and help guiding surveillance of complications. This concept is exemplified in Fig. 7.4.

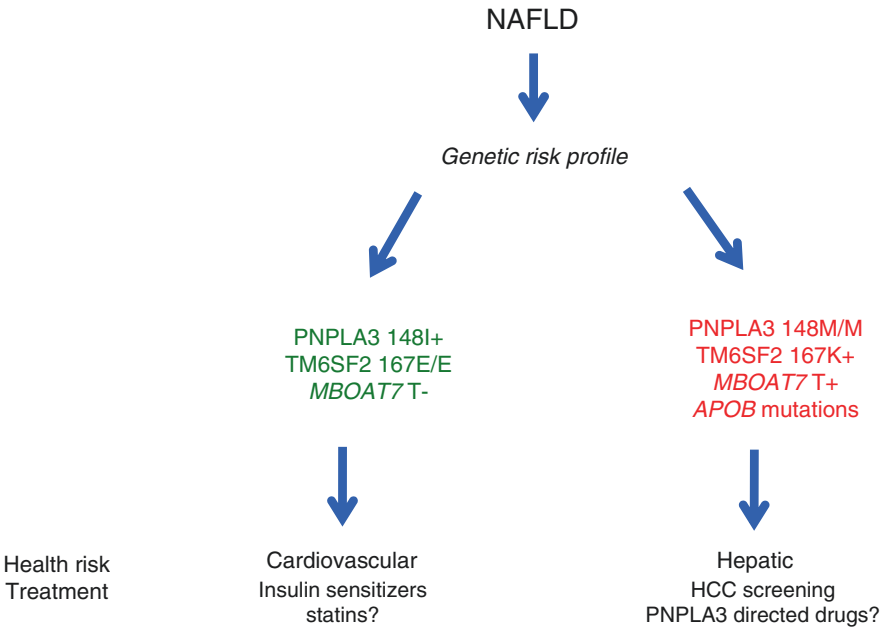

Fig. 7.4 Possible role of genetics for stratification of patients with NAFLD in those at higher risk of hepatic vs. cardiovascular complications, and disease management. *PNPLA3* patatin-like phospholipase domain-containing 3, *MBOAT7* membrane-bound O-acyl transferase 7, *TM6SF2* transmembrane 6 superfamily member 2, *APOB* apolipoprotein B

Carriage of specific genetic risk factors may influence the response and in particular the side effect of drugs. For example, the *PNPLA3* I148M variant has been reported to reduce the protective effect of statins on the risk of progressive NAFLD [93], to reduce the beneficial impact of dapagliflozin, an SGLT2 inhibitor, on hepatic fat accumulation, and to predict hepatotoxicity of glucagon receptor agonists and insulin peglispro, which is related to induction of hepatic fat accumulation [94–96]. Finally, drugs directly targeting protein mutated in NAFLD may prove beneficial to prevent progressive liver disease caused by fat accumulation. For example, silencing of the mutated PNPLA3 protein may potentially revert liver damage in carriers of the mutation by restoring dismissal of lipids from intracellular droplets, and possibly retinol metabolism [97]. This concept is presented in Fig. 7.5. Therefore, it could be envisioned that evaluation of genetic risk variants may help guiding pharmacological therapy for the disease. The clinical utility of these approached remains to be demonstrated in future studies.

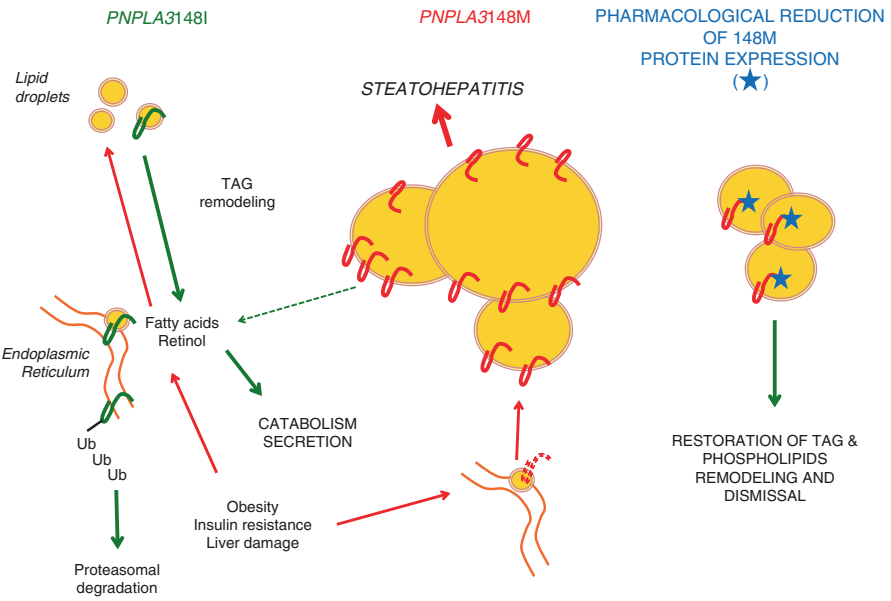

Fig. 7.5 Possible role of drugs silencing the mutated PNPLA3 in the prevention and treatment of liver disease in carriers of the I148M variant. Beneficial fluxes of lipids are indicated by green arrows, detrimental pathways as red arrows. The wild-type protein is involved in the remodelling and dismissal of lipids from lipid droplets under insulin resistance conditions. The mutated I148M variant is not enzymatically active and accumulate at the surface of lipid droplets because it is not ubiquitylated. At this level, it acquires the ability to impede lipid remodelling causing their retention and likely reduced turnover and activation of lipotoxicity. Pharmacological approaches that downmodulate the PNPLA3 I148M protein may contrast this pathophysiological mechanism. *PNPLA3* patatin like phospholipase domain-containing 3, *TAG* triglycerides, *Ub* ubiquitin

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NAFLD, Diabetes, and Other Endocrine Diseases: Clinical Implications

8

Giovanni Targher and Alessandro Mantovani

8.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is a metabolic liver disease that encompasses a spectrum of progressive pathologic conditions, ranging from simple steatosis to steatohepatitis (NASH), fibrosis, and cirrhosis. NAFLD is the most common liver disease in high-income countries affecting at least 25% of the general adult population. This liver disease affects up to 70–80% of patients with type 2 diabetes mellitus (T2DM) and up to 30–40% of adults with type 1 diabetes mellitus (T1DM) [1–3].

It is well known that NAFLD and T2DM often coexist and may act synergistically to drive adverse hepatic and extrahepatic clinical outcomes [1–3]. However, the link between NAFLD and T2DM is more complex than previously thought. It is now becoming clear that there is a close, bi-directional relationship between NAFLD and T2DM, and that NAFLD may also precede and/or promote the development of incident T2DM [4].

Abnormalities in various endocrine axes have been also associated with NAFLD [5]. In addition to diabetes, NAFLD is often present in patients with other common endocrine diseases, such as polycystic ovary syndrome (PCOS) and primary hypothyroidism [6–8]. NAFLD may be also present in patients with hypogonadism, growth hormone deficiency, acromegaly, or Cushing's syndrome, but the associations between NAFLD and these less frequent endocrine diseases have not been extensively explored in large series of patients [6].

It is possible to assume that the significant associations between NAFLD and common metabolic and endocrine diseases might also shed light in the aetiological

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mechanisms underpinning the pathogenesis of NAFLD. Moreover, understanding the hormonal regulation of NAFLD might lead to advances in the pharmacological treatment of this liver disease in the near future.

This chapter focuses on the significant relationships of NAFLD with type 1 and type 2 diabetes and other two common endocrine diseases (i.e., PCOS and primary hypothyroidism), and the adverse effects of NAFLD on the risk of developing chronic vascular complications of diabetes (mainly cardiovascular disease and chronic kidney disease).

NAFLD is an increasingly prevalent and burdensome liver disease that has been often overlooked by diabetologists and endocrinologists. Therefore, the major aim of this chapter is to not only to examine the rapidly expanding body of clinical evidence that supports a strong association of NAFLD with diabetes and other common endocrine diseases but also to raise awareness within the endocrine/gastroenterology community.

8.2 Epidemiological Evidence Linking NAFLD to Polycystic Ovary Syndrome

PCOS is a complex endocrine disorder that affects a significant proportion of women of reproductive age (affecting up to nearly 10% of these women) in the Europe and worldwide [9]. PCOS is one of the leading causes of fertility problems in women, and can lead to additional health problems in later life (i.e., with increased rates of T2DM, hypertension, and cardiovascular events). Women with PCOS have hyperandrogenism (clinical, biochemical, or both), ovulatory dysfunction, and polycystic ovarian morphologic features; additionally, these women are often overweight or obese and have greater insulin resistance [9].

To date, several cross-sectional and case–control studies have assessed the relationship between PCOS and NAFLD (for review see [7]). In most of these published studies, PCOS was diagnosed using the Rotterdam criteria (i.e., the most widely used criteria for diagnosing the disease) [9], except for few studies, which used other diagnostic criteria. Over a dozen cross-sectional studies showed that the prevalence of NAFLD (mostly detected by ultrasonography) is markedly increased in young women with PCOS, independent of overweight/obesity and other metabolic syndrome features. In these studies, the prevalence of NAFLD in women with PCOS ranges from approximately 35 to 70% compared with approximately 20 to 30% in age- and body mass index (BMI)-matched control women [7].

A comprehensive meta-analysis of 17 case–control studies published through 2017 (involving a total of approximately 2700 women with PCOS and 2600 matched control women) confirmed that PCOS women had a ~2.5-fold increased rate of NAFLD (fixed-effects odds ratio 2.25, 95% CI 1.95–2.60; I^2 = 5%) compared to control women, irrespective of age and BMI [10]. In addition, PCOS women with hyperandrogenism had a significantly higher risk of having NAFLD than controls. Conversely, normo-androgenic PCOS women did not seem to have

a higher prevalence of NAFLD when compared to controls [10]. Similar results were observed in another recent meta-analysis [11]. Accordingly, in a small case–control study involving 29 obese women with PCOS and 22 healthy controls who were matched for age, BMI, and waist circumference, Jones et al. found that hyper-androgenic PCOS women had a significantly higher intrahepatic fat content on magnetic resonance spectroscopy compared to both normo-androgenic PCOS women and matched controls (mean intrahepatic fat content: 12.9% vs. 0.6% vs. 1.9%, respectively) [12]. In a case–control study of 275 young nonobese women with PCOS and 892 nonobese control women, Kim et al. found that the prevalence of ultrasound-diagnosed NAFLD was significantly greater in women with PCOS than in controls (5.5% vs. 2.8%), and that the presence of hyperandrogenemia (i.e., higher levels of free testosterone or free androgen index) was significantly associated with NAFLD even after adjustment for age, BMI, plasma lipid profile, insulin resistance, or glycemic status [13]. More recently, Kumarendran et al. performed a population-based retrospective cohort study utilizing a large UK primary care database and included more than 63,000 women with PCOS and 121,000 matched controls registered between 2000 and 2016. Notably, these authors found that rates of NAFLD were significantly increased in women with PCOS (even after adjusting for BMI and dysglycemia), and identified androgen excess as a potential additional contributing risk factor for NAFLD development in PCOS [14].

All these findings suggest that androgen excess might represent a possible causative mechanism linking PCOS to the development and progression of NAFLD (in addition to coexisting abdominal obesity and insulin resistance). However, future larger studies are needed to determine if androgen excess also drives the progression of NAFLD to liver inflammation and fibrosis, and to establish whether anti-androgen treatment may reduce the risk of NAFLD.

Notably, some small case–control studies performed at tertiary gastroenterology centers showed that PCOS is also a common pathologic condition in patients with biopsy-proven NAFLD [15–17]. Among these patients the prevalence of PCOS ranged from approximately 50% to 70%, and these women were also more likely to develop the more severe histologic forms of NAFLD (i.e., NASH with varying degrees of fibrosis on liver histology) [15–17].

Collectively, although further well-conducted studies on larger series of carefully characterized women with PCOS are needed to corroborate these findings and to better elucidate the biological mechanisms underlying the association between PCOS and NAFLD, the aforementioned studies clearly indicate that the prevalence of NAFLD is significantly higher in women with PCOS than in control women, independent of age, overweight/obesity, and other coexisting metabolic syndrome features. Furthermore, the young age of many women with PCOS and the relatively advanced stage of NASH (as revealed by liver biopsies from these patients) clearly suggest the possibility of an increased risk for long-term liver-related complications in this group of patients over the course of their lives. Therefore, we believe that the currently available literature argues for a systematic screening for NAFLD in young women with PCOS (especially in those with PCOS-related androgen excess).

8.3 Epidemiological Evidence Linking NAFLD to Primary Hypothyroidism

Overt primary hypothyroidism is an endocrine disorder affecting up to nearly 3–4% of individuals living in iodine-replete communities that is defined by insufficient levels of serum thyroid hormones. Primary hypothyroidism has multiple aetiologies and manifestations [18].

It is known that the development and progression of NAFLD usually occur in the presence of profound derangements of lipid and glucose metabolism, and dysregulation of energy homeostasis [1, 2, 5]. Thyroid hormones are critical regulators of energy homeostasis and have prominent direct effects on lipid and glucose metabolism [19].

To date, several observational studies have explored the association between primary subclinical/overt hypothyroidism and imaging-defined or biopsy-proven NAFLD [20]. However, the findings from these studies have been conflicting so far, with some studies reporting that the prevalence of primary hypothyroidism, especially subclinical hypothyroidism, was extremely common among patients with NAFLD (occurring in up to 20–25% of these patients) [21, 22], while other studies failing to find any significant association between primary hypothyroidism and risk of NAFLD [23–25]. On this background of evidence, it remains uncertain whether subclinical hypothyroidism is a risk factor for NAFLD.

Recently, we carried out a systematic review and meta-analysis of observational, cross-sectional and longitudinal studies examining the association between primary hypothyroidism and risk of NAFLD [26]. This meta-analysis involved a total of 15 observational studies using either liver biopsy or imaging techniques (mostly ultrasonography) to diagnose NAFLD with aggregate data on 44,140 individuals with nearly 15% of them who were either taking levothyroxine replacement therapy or had either subclinical or overt hypothyroidism based on thyroid function tests [26]. As shown in Fig. 8.1, meta-analysis of data from the 12 cross-sectional studies has shown that the presence of variably defined hypothyroidism was significantly associated with a 42% increased risk of imaging-defined or biopsy-proven NAFLD (n = 12 studies; random-effects odds ratio 1.42, 95% CI 1.15–1.77; I^2 = 51.2%), independently of age, sex, BMI, diabetes, or metabolic syndrome. This risk tended to increase across the different definitions used for diagnosing hypothyroidism (i.e., a self-reported history of hypothyroidism with use of levothyroxine replacement therapy > newly diagnosed overt biochemical hypothyroidism > newly diagnosed subclinical hypothyroidism), and appeared to further increase with greater histologic severity of NAFLD (Fig. 8.2). Conversely, meta-analysis of data from the three longitudinal studies has shown that subclinical hypothyroidism was not significantly associated with the risk of incident NAFLD (assessed by ultrasonography) over a median follow-up of 5 years (n = 3 studies; random-effects hazard ratio 1.29, 95% CI 0.89–1.86; I^2 = 83.9%), after adjusting for age, sex, BMI, diabetes, or other known metabolic risk factors [26]. However, on the basis of these three longitudinal studies included in the meta-analysis [23, 27, 28], it is likely that this finding could be due to the lack of adequate statistical power, and that larger

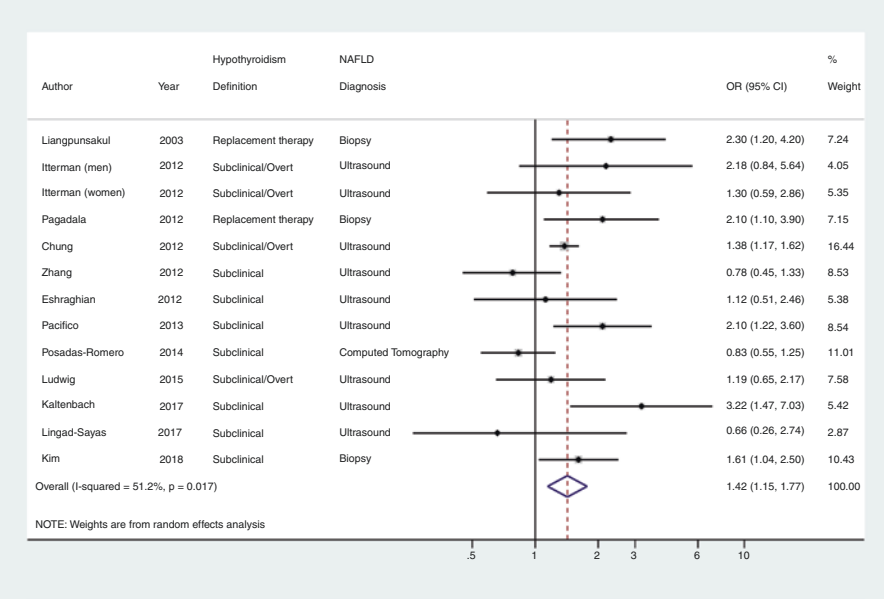

Fig. 8.1 Forest plot and pooled estimates of the effect of variably defined primary hypothyroidism (defined as either self-reported use of levothyroxine replacement therapy or abnormal concentrations of serum thyroid stimulating hormone and/or free thyroxine) on the risk of prevalent NAFLD in 12 eligible cross-sectional studies. (Reproduced with permission [26])

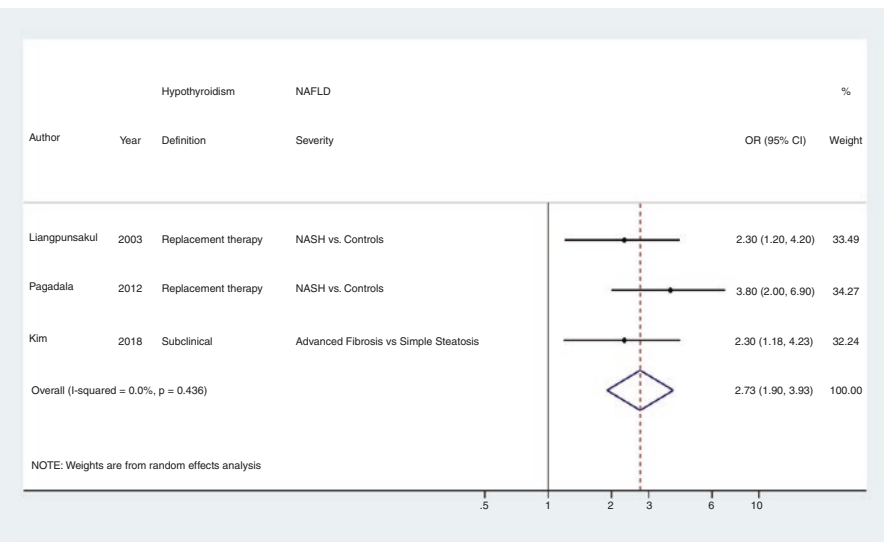

Fig. 8.2 Forest plot and pooled estimates of the effect of variably defined primary hypothyroidism on the severity of NAFLD on liver histology in three eligible cross-sectional studies. (Reproduced with permission [26])

(n > 10,000 individuals) prospective cohort studies with longer follow-up periods (≥10 years) will be needed to better elucidate this important topic. As expected, no sufficient data were available in most of the studies included in the meta-analysis to examine the effect of newly diagnosed overt hypothyroidism on the risk of developing incident NAFLD. Nevertheless, it should also be noted that Bano et al. found that both subclinical and overt hypothyroidism were independently associated with an increased 10-year risk of developing incident NAFLD with clinically significant hepatic fibrosis (assessed by Fibroscan®) in a large population-based cohort of elderly Dutch individuals [28].

Collectively, we believe that the findings of this updated meta-analysis support the view that the presence of variably defined primary hypothyroidism is significantly associated with NAFLD, and may also have clinical practice implications for the potential screening of hypothyroidism and NAFLD. Indeed, these findings suggest that patients with NAFLD should probably be screened for primary hypothyroidism (a disease necessitating hormone replacement therapy); and that NAFLD should be looked for in patients with hypothyroidism, given that these patients are at higher risk of having NASH and advanced fibrosis.

However, on the basis of the currently available literature, it should also be noted that the temporal relationship between liver and thyroid diseases is not clear, and that a causal relationship between NAFLD and primary hypothyroidism cannot be definitely established [26]. Again, it should be noted that the levels of thyroid antibodies were not consistently measured in any of the aforementioned studies (except for the study by Bano et al. [28], who measured serum thyroid peroxidase antibodies, but did not find any significant association between levels of thyroid peroxidase antibodies and risk of incident NAFLD); hence, the cause of hypothyroidism is not clear. In addition, none of the included studies examined the effects of levothyroxine replacement therapy when exploring the risk of NAFLD in patients with subclinical or overt hypothyroidism. Further large prospective studies to confirm these findings should be undertaken, and mechanistic studies to better elucidate the mechanisms underlying the association between hypothyroidism and NAFLD are also warranted.

A detailed description of the multifactorial pathogenesis involved in the hypothyroidism-induced NAFLD is beyond the scope of this chapter. To date, however, there is convincing evidence of biological plausibility that overt hypothyroidism can promote the development of NAFLD through multiple extrahepatic and intrahepatic mechanisms [19, 20]. Indeed, hypothyroidism can induce NAFLD through the systemic development of metabolic disorders, low-grade inflammation, and increased oxidative stress [19, 20]. Moreover, thyroid hormones also have direct effects on hepatic lipid and glucose metabolism [19]. In addition to the adverse effects of decreased serum thyroid hormones on hepatic glucose and lipid metabolism, it is also possible that increased serum TSH levels *per se* could promote the development of NAFLD by stimulating hepatic *de novo* lipogenesis [29]. Additionally, the intrahepatic thyroid hormone concentration and/or thyroid hormone signaling could be decreased in the livers of patients with NAFLD [20, 30]. On this background of evidence, it is possible to assume that thyroid hormone analogs or mimetics could be useful for the treatment of NAFLD [19, 20]. In a

recent phase 2b single-arm trial performed in six hospitals in Singapore, it has been reported that low-dose levothyroxine significantly decreased intrahepatic lipid content (with a relative reduction of 12% of IHLC as measured by magnetic resonance spectroscopy) in 20 euthyroid patients with type 2 diabetes and NAFLD [31]. A double-blinded, randomized, placebo-controlled phase 2 trial is also ongoing to evaluate the efficacy and safety of MGL-3196, i.e., a selective liver-directed, thyroid hormone receptor-β agonist, in patients with biopsy-proven NASH.

8.4 Epidemiological Evidence Linking NAFLD to Diabetes Mellitus

NAFLD and diabetes mellitus are common diseases that often coexist and act synergistically to drive adverse hepatic and extrahepatic clinical outcomes [1–3, 32]. The coexistence of NAFLD and diabetes increases the risk of developing both microvascular and macrovascular complications of diabetes as well as increasing the risk of developing more severe forms of NAFLD (as extensively reviewed in [32]). In addition, patients with NAFLD and diabetes have an increased risk of all-cause and cause-specific (cardiovascular, cancer, and liver) mortality compared to those without NAFLD [1, 2, 32].

8.4.1 Prevalence of NAFLD in Diabetes and Risk of Liver-Related Complications

Patients with established T2DM have a high prevalence of NAFLD. Indeed, in these patients imaging-diagnosed NAFLD ranges from approximately 45 to 75% in large hospital-based studies and from 30 to 70% in population-based studies [32]. For example, in the Valpolicella Heart Diabetes Study, involving nearly 2800 Italian outpatients with T2DM (mean age: 63 years, mean BMI: 27 kg/m^2), the prevalence of NAFLD on ultrasonography was nearly 70% [33].

In patients with T2DM, the coexistence of NAFLD is associated with poorer glycemic control, more severe hyperinsulinemia, and greater insulin resistance in the skeletal muscle, adipose tissue, and liver compared with their counterparts without NAFLD [34]. In clinical practice, it is well established that T2DM patients with NAFLD have a poorer quality of glycemic control and require a higher daily amount of insulin to get a good glycemic control than their counterparts without NAFLD [34]. It is believed that increased intrahepatic fat accumulation is an important determinant of insulin resistance in the liver and affects both the daily dosage of glucose-lowering therapy and the achieving good glycemic control in patients with T2DM [32, 34]. These considerations suggest that treatment strategies that decrease intrahepatic fat accumulation and improve insulin sensitivity might partly contribute to improved glycemic control in patients with T2DM and NAFLD.

Substantial evidence indicates that people with T2DM are also at higher risk of developing NASH, and a twofold to fourfold higher risk of developing serious

liver-related complications, such as cirrhosis, liver failure, and hepatocellular carcinoma [1, 2, 32, 35, 36]. It is also notable that, in dual biopsy studies, the development of incident T2DM was the strongest clinical predictor of faster progression to NASH, advanced fibrosis and cirrhosis [37].

A recent study that used magnetic resonance imaging to assess hepatic fat content and magnetic resonance elastography (MRE) to estimate liver stiffness has reported high rates of both hepatic steatosis (defined as MRI-PDFF ≥5%) and advanced fibrosis (defined as MRE ≥3.6 kPa) in a cohort of 100 consecutive patients with T2DM in primary care, who did not have any other aetiology of liver disease (i.e., 65% of these patients had hepatic steatosis and 7.1% had advanced fibrosis, respectively) [38]. A high prevalence of NAFLD and advanced fibrosis was also reported in a hospital cohort of 1918 Chinese adult patients with T2DM (mean age: 60.6 years, mean BMI: 26.6 kg/m^2) where hepatic fat and fibrosis were simultaneously assessed with FibroScan® (i.e., ~73% of them had CAP ≥222 dB/m and 17.7% had LSM ≥9.6 kPa, respectively) [39]; notably, as shown in Fig. 8.3, in a subset of these patients with T2DM submitted to liver biopsy (n = 94), 56% had NASH and 50% had advanced fibrosis [39]. In the NASH-Clinical Research Network cohort study enrolling nearly 1300 US adult patients with biopsy-proven NAFLD, the authors found that the prevalence of NASH and advanced fibrosis in the subgroup of those with T2DM and NAFLD (n = 346; mean age: 53 years, mean

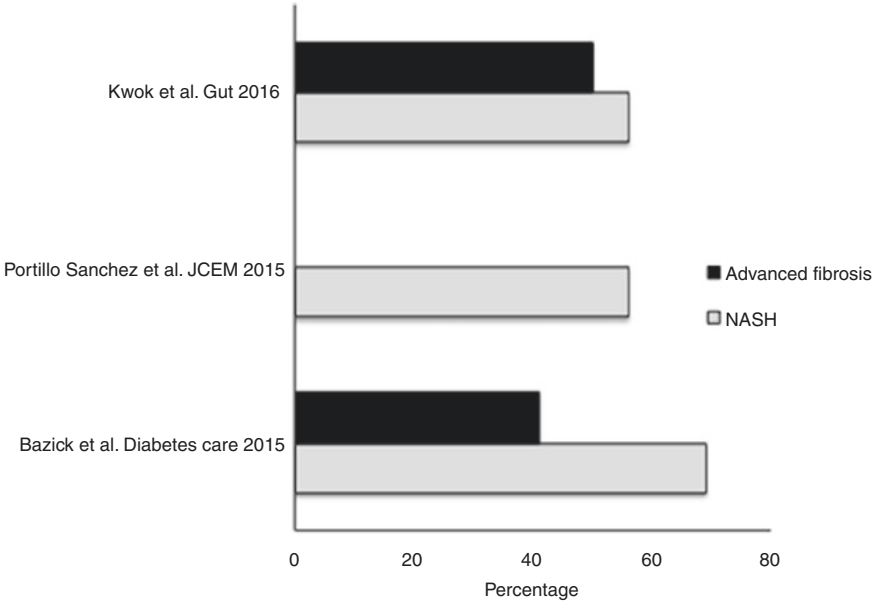

Fig. 8.3 Prevalence of NASH and advanced fibrosis on liver histology in patients with type 2 diabetes (irrespective of serum aminotransferase concentrations). (Data are derived from studies published by Kwok et al. [39], Bazick et al. [40], and Portillo-Sanchez et al. [41], respectively. Reproduced with permission [4])

BMI: 35.8 kg/m^2) was 69.2% and 41%, respectively [40]. Similarly, the prevalence of NASH was found to be as high as 56% in a small study of obese patients with T2DM and normal serum aminotransferase concentrations (n = 103; mean age: 60 years, mean BMI: 33 kg/m^2) [41]. Notably, a large administrative health database (involving almost 2.5 million people) documented that Canadian adults with newly diagnosed T2DM had an approximately twofold higher risk of developing cirrhosis, liver failure, or liver transplantation than matched individuals without diabetes over a follow-up period of 12 years [42]. Finally, prospective studies have shown that there is also a strong link among T2DM, NAFLD/NASH, and risk of hepatocellular carcinoma (HCC) [43, 44]. In fact, the coexistence of T2DM increases the risk of developing HCC (approximately from 1.5 to 4-fold) [35, 36, 43, 44]. Preclinical and observational studies also suggested that hypoglycemic agents can modulate the risk of incident HCC in patients with T2DM [45, 46]. However, the effect of each individual hypoglycemic agent should be interpreted cautiously owing to inherent cancer-modifying effect of the comparator group. Further large randomized clinical trials are needed to confirm these findings.

Worryingly, it is also well known that the coexistence of NAFLD may also adversely influence the prognosis of diabetes [32, 35]. Using the electronic administrative database of death certificates of the Veneto Region (Northern Italy), Zoppini et al. found that people with diabetes (n = 167,621 diabetic individuals aged 30–89 years) had a nearly threefold higher risk of dying of chronic liver diseases, mainly due to NAFLD [47]. In line with these findings, Adams et al. found that the coexistence of NAFLD (diagnosed by imaging or biopsy) carried an approximately twofold increased risk of all-cause mortality (mainly due to cardiovascular disease, malignancy, and liver-related complications) over a mean follow-up of 11 years in a community-based cohort of 337 residents of Olmsted County, Minnesota, with diabetes mellitus [48]. Again, a national cohort study of Scottish people aged 40–89 years documented that NAFLD was the most common liver disease in people with T2DM, and that T2DM was closely associated with an increased risk of hospital admissions or death for NAFLD [49].

All the aforementioned considerations fully support a screening for NAFLD in patients with established T2DM, and the need for close and intensive surveillance for advanced liver disease in those with NAFLD [1, 3, 32]. It is also reasonable to assume that an early diagnosis and treatment of NAFLD, if any, may have a beneficial clinical impact on survival rates of patients with T2DM.

At present, few data are available regarding the prevalence and natural history of NAFLD in patients with T1DM. However, the epidemiological impact of both NAFLD and the metabolic syndrome seems to be relevant also in adult patients with T1DM since the prevalence of the metabolic syndrome is steadily growing in these patients, being nowadays approximately of 40% [32]. Although there are conflicting results, some studies reported that NAFLD on ultrasonography is present in approximately 30–40% of adult patients with T1DM [32, 50]. In a longitudinal cohort of T1DM and T2DM patients who undergone a liver biopsy, it was demonstrated that adult patients with T1DM had a high risk of developing cirrhosis and portal hypertension, and that this risk was even comparable with that observed in

patients with T2DM, who were matched for duration of diabetes, obesity, and other comorbidities [51]. However, further studies are required to better characterize the relationship between NAFLD and T1DM.

8.4.2 NAFLD and Risk of Chronic Kidney Disease and Other Microvascular Complications

Accumulating epidemiological evidence indicates that the presence of imaging-diagnosed NAFLD is associated with an increased risk of microvascular complications of diabetes, such as chronic kidney disease (CKD), retinopathy, and distal symmetric polyneuropathy [32].

For instance, in a large cohort study involving 2103 ambulatory patients with T2DM, it has been reported that those with ultrasound-diagnosed NAFLD had remarkably higher age- and sex-adjusted prevalence rates of both non-proliferative and proliferative/laser-treated retinopathy and CKD than patients without NAFLD. Logistic regression analysis showed that NAFLD was associated with increased rates of CKD (adjusted-odds ratio 1.87; 95% CI 1.3–4.1) and proliferative/laser-treated retinopathy (adjusted-odds ratio 1.75; 95% CI 1.1–3.7), even after adjustment for multiple cardiometabolic risk factors, diabetes-related variables, and other potential confounders [52]. Other studies have clearly shown that the presence and severity of NAFLD was associated with an increased prevalence of abnormal albuminuria or decreased kidney function in patients with T2DM or pre-diabetes [32, 53]. Similarly to what reported in T2DM patients, some studies also showed that NAFLD was independently associated with a higher prevalence of both CKD and diabetic retinopathy in adult patients with T1DM [54].

To date, there is a paucity of published data regarding the risk of developing CKD in diabetic patients with NAFLD. The Valpolicella Heart Diabetes Study showed that patients with T2DM and NAFLD had a higher risk of developing incident CKD (i.e., CKD stage ≥3 with/without accompanying overt proteinuria) compared with their counterparts without NAFLD over a mean follow-up period of 6.5 years. Notably, this risk remained significant even after adjusting for a broad range of coexisting cardio-renal risk factors (including also diabetes duration, hemoglobin A1c, hypertension, baseline e-GFR, albuminuria, and current use of medications) [55].

In line with this finding, in a small follow-up cohort study involving 261 T1DM adult patients with preserved kidney function and without overt proteinuria at baseline, who were followed for a mean period of 5.2 years, the presence of ultrasound-diagnosed NAFLD was independently associated with an increased incidence of CKD (hazard ratio 2.85, 95% CI 1.6–5.1). Notably, addition of NAFLD to traditional cardio-renal risk factors significantly improved the discriminatory capability of the regression models for predicting CKD [56].

We recently performed a systematic review and meta-analysis of nine observational studies, involving 96,595 adult individuals (~34% with either imaging-diagnosed or biochemistry-based NAFLD) of predominantly Asian descent and nearly 5000 cases of incident CKD (i.e., CKD stage ≥3) over a median follow-up

of 5.2 years [57]. No studies with biopsy-proven NAFLD were available in the literature for the analysis. As shown in Fig. 8.4, patients with imaging-diagnosed NAFLD had a significantly higher long-term risk of incident CKD compared with those without NAFLD (pooled random-effects hazard ratio 1.37, 95% CI 1.20–1.53; $I^2 = 33.5\%$), even after adjustment for common cardio-renal risk factors [55]. Patients with more "severe" NAFLD (according to ultrasonographic steatosis scores or non-invasive biomarkers of liver fibrosis) were also more likely to develop incident CKD (random-effects hazard ratio 1.50, 95% CI 1.25–1.74; $I^2 = 0\%$). Interestingly, as also shown in Fig. 8.4, when the analysis was stratified by the study population, the association between NAFLD and the risk of incident CKD appeared to be stronger in studies that enrolled patients with established diabetes (random-effects hazard ratio 1.56, 95% CI 1.07–2.05; $I^2 = 0\%$) [57].

Finally, preliminary evidence also suggests that NAFLD is associated with an increased prevalence of distal symmetric polyneuropathy and cardiovascular autonomic neuropathy in patients with T1DM or T2DM [58, 59]. However, further research is needed to confirm these data.

Despite the growing evidence of biological plausibility linking NAFLD with CKD and other microvascular complications in adult patients with T1DM or T2DM,

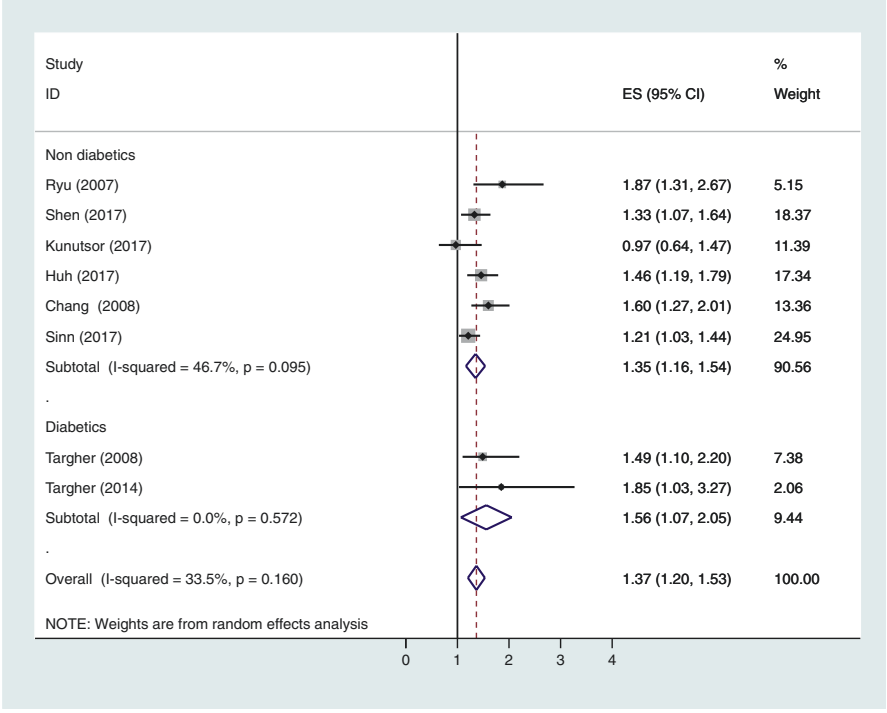

Fig. 8.4 Forest plot and pooled estimates of the effect of NAFLD on the risk of incident chronic kidney disease (CKD stage ≥3) in eight eligible prospective studies, stratified by study population (diabetes vs. no-diabetes). (Reproduced with permission [57])

it still remains to be definitively established whether a causal association also exists [60]. Additional prospective and mechanistic studies are needed to better elucidate the independent contribution of NAFLD to the increased risk of developing microvascular diabetic complications in patients with NAFLD. In the meantime, however, all the aforementioned studies provide further support for the view that a diagnosis of NAFLD identifies a subset of individuals, who are at higher risk of incident CKD (stage ≥3), and who need more intensive surveillance and early treatment to decrease the risk of developing CKD [32, 60].

8.4.3 NAFLD and Risk of Macrovascular Complications

Strong evidence indicates that cardiovascular disease dictates the outcome(s) in patients with NAFLD more frequently and to a greater extent than does the progression of liver disease in both patients with and without diabetes [2–4, 61, 62]. Recent cohort studies of patients with histologically confirmed NAFLD have clearly demonstrated that cardiovascular disease is the leading cause of mortality in these patients (~40–45% of the total deaths), and that fibrosis stage is the strongest histologic predictor for overall and disease-specific mortality in NAFLD [63].

Several cross-sectional studies have consistently shown that NAFLD was closely associated with both various markers of subclinical atherosclerosis and clinically manifest CVD across a wide range of patient populations, including also patients with diabetes [4, 61, 62]. For example, in the Valpolicella Diabetes Heart Study, it has been reported that type 2 diabetic patients with NAFLD (detected by ultrasonography) had a remarkably higher prevalence of clinically manifest coronary, cerebrovascular, and peripheral vascular disease compared to their counterparts without NAFLD, even after adjustment for traditional cardiovascular risk factors, hemoglobin A1c, use of medications, and other important diabetes-related confounders [33]. Almost identical findings were also reported in adult patients with T1DM [50].

Notably and most importantly, a number of hospital-based and population-based studies also reported that NAFLD (diagnosed by imaging techniques) was significantly associated with an increased incidence of fatal and nonfatal cardiovascular events, independent of established cardiovascular risk factors, both in patients with T2DM and in those without T2DM (as extensively reviewed in [4, 62]).

For instance, the Valpolicella Diabetes Heart Study documented that patients with T2DM and NAFLD (who were free from prior cardiovascular disease at baseline) had a nearly twofold increased risk of developing nonfatal ischemic heart disease, ischemic stroke, or cardiovascular death compared with patients without NAFLD over a 6.5-year follow-up period [64]. Notably, this relationship was independent of traditional cardiovascular risk factors, diabetes duration, hemoglobin A1c, and use of hypoglycemic, anti-hypertensive, lipid-lowering, and antiplatelet drugs [64]. Similarly, in a retrospective cohort of 286 adult patients with T1DM, who were followed for a mean period of 5.3 years for the occurrence of incident CVD events (i.e., a combined endpoint inclusive of nonfatal ischemic heart disease, ischemic stroke, or coronary/peripheral artery revascularizations), the presence of

NAFLD on ultrasonography was associated with an increased risk of incident CVD events, independent of established cardiovascular risk factors and diabetes-related variables [65].

A comprehensive meta-analysis that incorporated almost 34,000 individuals in 16 observational cohort studies concluded that the presence of NAFLD (diagnosed either by imaging methods or by histology) was significantly associated with a nearly 65% increased risk of developing fatal and nonfatal cardiovascular events over a median follow-up of 6.9 years (Fig. 8.5), and that this risk increased further with greater severity of NAFLD (defined either by presence of hepatic steatosis on imaging *plus* either increased serum gamma-glutamyltransferase concentrations or high NAFLD fibrosis score or high ^{18}F-fluoro-2-deoxyglucose uptake on positron emission tomography, or by increasing fibrosis stage on liver histology) [66].

Although the results of this updated meta-analysis strongly support the existence of a significant association between NAFLD and the risk of developing fatal and

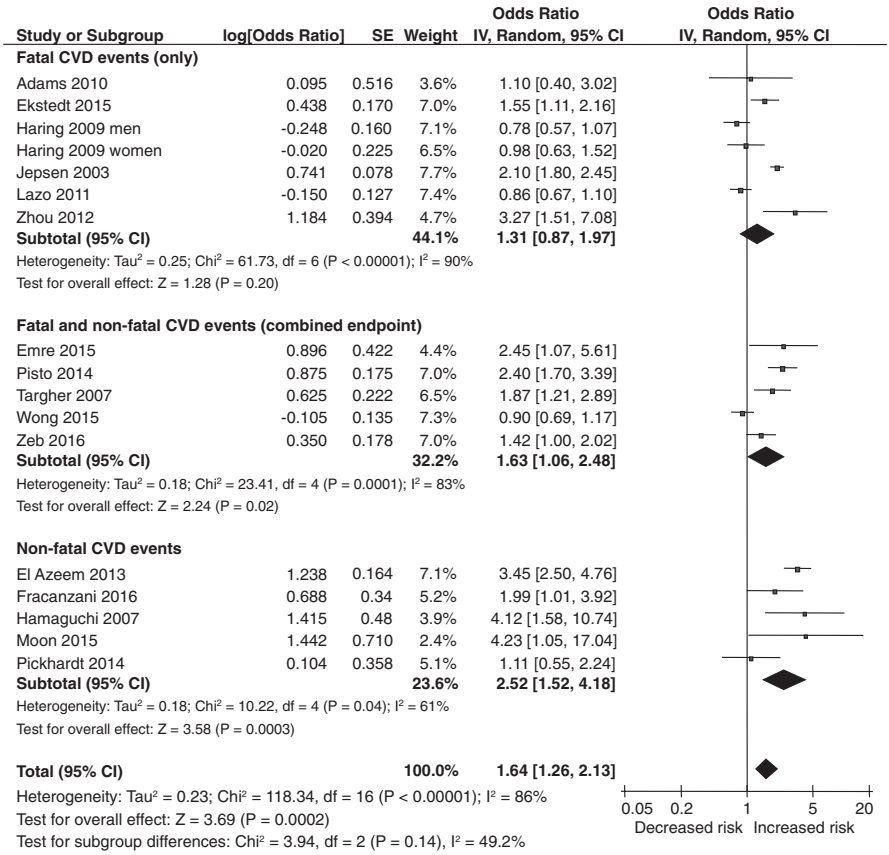

Fig. 8.5 Forest plot and pooled estimates of the effect of NAFLD on the risk of incident cardiovascular events (fatal, nonfatal, or both) in 16 eligible prospective studies. (Reproduced with permission [66])

nonfatal CVD events both in patients with and without diabetes, it is important to underline that the observational design of the eligible studies does not allow for proving causality [66]. Moreover, the key question of whether the prognostic role of NAFLD in the development of cardiovascular disease is restricted to NASH/advanced fibrosis or is also associated with simple steatosis remains partly unresolved. More research is needed to address this issue.

In the past few years, compelling evidence has also emerged for a strong association between NAFLD and risk of cardiomyopathy (mainly left ventricular diastolic dysfunction and hypertrophy, possibly leading to the development of congestive heart failure over time), cardiac valvular calcification (mainly aortic-valve sclerosis and mitral annulus calcification), cardiac arrhythmias (mainly permanent atrial fibrillation), and some cardiac conduction defects (mainly persistent first-degree atrio-ventricular block, right bundle branch block, and left anterior hemi-block) both in patients without diabetes and in those with T2DM [67]. All of these additional NAFLD-related heart diseases could further contribute to the increased risk of cardiovascular morbidity and mortality observed among patients with NAFLD.

A detailed description of the complex and multifactorial pathogenesis linking NAFLD to cardiovascular disease is beyond the scope of this chapter. However, there are a myriad of possible underlying mechanisms that plausibly link NAFLD to the development and persistence of coronary atherosclerosis, cardiomyopathy, cardiac arrhythmias, and certain cardiac conduction defects. Indeed, NAFLD, especially in its more advanced forms (NASH with varying amounts of liver fibrosis), exacerbates systemic and hepatic insulin resistance, predisposes to atherogenic dyslipidemia, and causes the release of multiple pro-inflammatory, pro-fibrogenic, and vasoactive mediators that can promote the development and progression of cardiovascular, cardiac, and arrhythmic complications [4, 61, 62, 67]. To date, however, it should be noted that no studies have definitely established a cause–effect relationship, and further research is required to gain mechanistic insights into the pathophysiology linking NAFLD to these cardiovascular, cardiac, and arrhythmic complications. Moreover, it is not yet established whether addition of NAFLD to the currently available risk assessment equations improves CVD risk prediction. Finally, since NAFLD is heterogeneous and may be also caused by common genetic variants (e.g., patatin-like phospholipase domain-containing 3 [PNPLA3] variants or trans-membrane 6 superfamily member 2 [TM6SF2] variants), it will be also interesting to ascertain whether obese/metabolic NAFLD and genetic-related NAFLD produce the same risk of developing cardiovascular events [4, 62, 67].

Collectively, we believe that the current evidence from the published studies clearly indicates that a diagnosis of NAFLD identifies a subset of individuals, which are exposed to at higher risk of fatal and nonfatal cardiovascular events. These findings further reinforce the notion that NAFLD is a *multisystem* disease that affects many extrahepatic organ systems, including the heart and vasculature, by disrupting the regulation of several metabolic and inflammatory pathways [4, 61, 62, 67]. This concept also implies that all individuals with NAFLD should undergo careful cardiovascular surveillance as recommended by the most recent European, American, and Italian clinical practice guidelines for the management of NAFLD [68–70]. A more accurate, patient-centered, team-based approach to the management and treatment

of individuals with NAFLD, based on a careful evaluation of related cardiometabolic risk factors and monitoring for cardiovascular and liver complications, will be needed.

8.4.4 NAFLD and Risk of Developing Type 2 Diabetes

The link between NAFLD and T2DM is more complex than previously thought. Accumulating evidence now suggests that there is a mutual and bi-directional relationship between NAFLD and T2DM, and that NAFLD may also precede and/or promote the development of T2DM [4, 35, 71].

A large and updated meta-analysis of 19 longitudinal studies (including nearly 300,000 individuals and approximately 16,000 new cases of incident diabetes) confirmed that patients with imaging-defined NAFLD had a 2.2-fold increased risk of developing incident diabetes than those without NAFLD over a median follow-up of 5 years (Fig. 8.6), even after adjustment for age, sex, adiposity measures, and

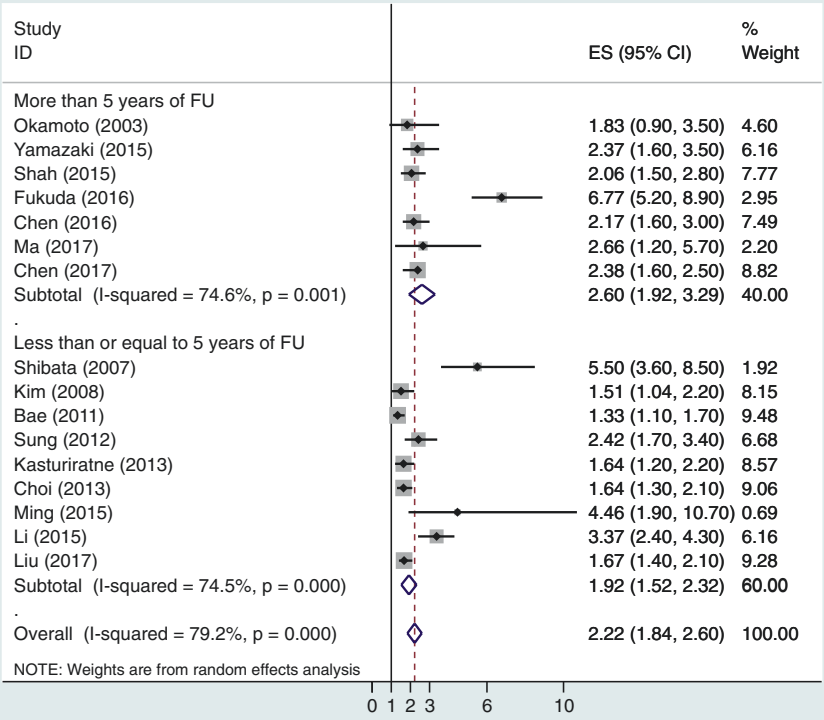

Fig. 8.6 Forest plot and pooled estimates of the effect of NAFLD on the risk of incident diabetes in 16 eligible studies, stratified by duration of follow-up (based on the median follow-up of the eligible studies). (Reproduced with permission [72])

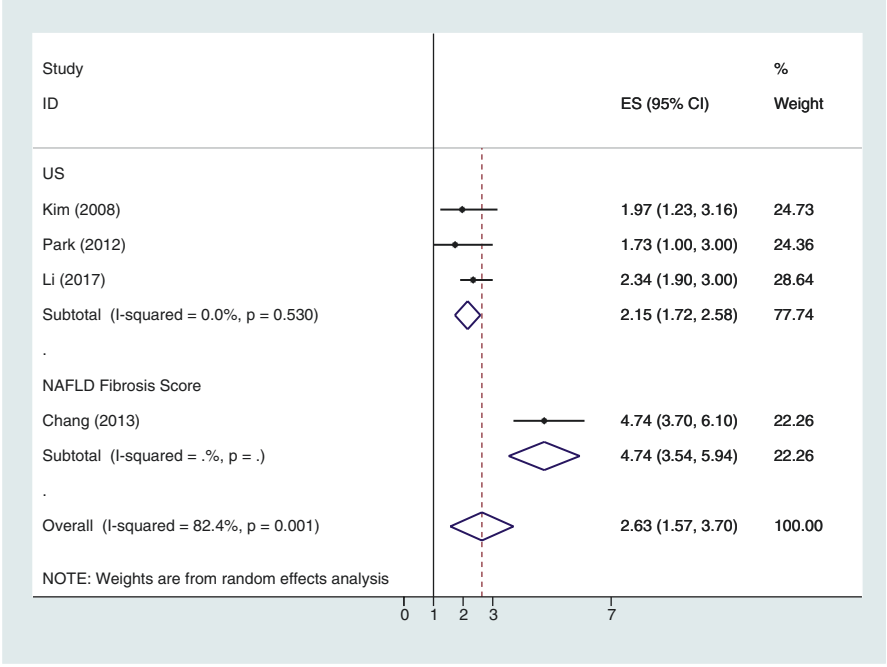

Fig. 8.7 Forest plot and pooled estimates of the effect of the severity of NAFLD (defined by ultrasonography [US] or high NAFLD fibrosis score [NFS]) on the risk of incident diabetes in four eligible studies. (Reproduced with permission [72])

other common metabolic risk factors [72]; the magnitude of this risk paralleled the underlying severity of NAFLD based on ultrasonographic steatosis scores and non-invasive biomarkers of fibrosis (Fig. 8.7) [72].

Notably, some large Asian cohort studies also showed that the risk of incident T2DM appears to diminish over time following the improvement or resolution of NAFLD on ultrasonography, adding weight to causality and suggesting that liver-focused treatments might reduce risk of developing some important extrahepatic complications of NAFLD [73, 74].

To date, there is convincing evidence regarding the biological plausibility of the role of NAFLD in the development of incident T2DM. Indeed, NAFLD, especially in its more severe histologic forms, may interact with the regulation of multiple metabolic pathways, and may be involved in the development of incident T2DM possibly via its direct contribution to hepatic insulin resistance and the systemic release of multiple hepatokines (e.g., fetuin-A, fetuin-B, retinol binding protein-4, selenoprotein P) that may adversely affect glucose metabolism and insulin action [4, 35, 72, 75].

However, it remains currently uncertain whether NAFLD is causally related to the development of incident T2DM or is simply a marker of other shared metabolic risk factors, such as expended visceral adipose tissue. Further large prospective studies are also needed in non-Asian populations, as most of the published studies have been conducted in Asian populations (especially in South Korean

people), where large populations undergo regular health check-ups, including liver ultrasonography. Finally, additional prospective studies are also required to establish whether adding NAFLD to the currently available algorithms will improve risk prediction for diabetes.

Despite the abovementioned caveats, there is now increasing evidence suggesting that NAFLD is associated with an approximate doubling of risk of incident T2DM. This association appears to be dose-dependent and is ameliorated with improvement or resolution of NAFLD over time. Consequently, current clinical guidelines do recommend routine screening of NAFLD patients for T2DM with fasting plasma glucose and hemoglobin A1c levels, or with a 75-g oral glucose tolerance test in high-risk patient groups [68–70].

8.5 Conclusions

NAFLD is a multisystem disease that affects many extrahepatic organ systems by disrupting the regulation of multiple metabolic and inflammatory pathways [62, 76]. It is important that clinical endocrinologists/diabetologists recognize the presence of NAFLD and its potentially devastating hepatic and extrahepatic consequences.

These clinicians have to keep in mind that NAFLD is very common in patients with T2DM and T1DM (affecting about 70–80% of those with T2DM and up to 30–40% of adult patients with T1DM), and that these patients are also more likely to develop the more severe histological forms of NAFLD (i.e., NASH, cirrhosis, and HCC). In addition, because of the close link between diabetes, NAFLD, and adverse vascular complications, more careful surveillance of these at-risk patients will be needed. Therefore, a more accurate, patient-centered, multidisciplinary-team-based approach to the management and treatment of diabetic patients with NAFLD, based on a careful evaluation of related cardiometabolic risk factors and monitoring for cardiovascular, kidney, and liver complications, is warranted.

Accumulating evidence suggests that NAFLD is a frequent condition also in patients with other common endocrine diseases, such as PCOS and primary hypothyroidism. Worryingly, these patients seem to be also more likely to develop NASH and advanced fibrosis. Although the observational design of the available studies does not allow for proving causality, and more mechanistic studies are required to better clarify the underlying mechanisms linking NAFLD to PCOS and primary hypothyroidism, we believe that the currently available literature argues for a systematic screening for NAFLD both in young women with PCOS (especially in those with PCOS-related androgen excess) and in patients with primary hypothyroidism. It is plausible to hypothesize that a better understanding of both the hormonal regulation(s) of NAFLD and the links of NAFLD with these common endocrine diseases will also result in future advances in the pharmacological treatment of this increasingly prevalent and burdensome liver disease.

Conflict of Interest All authors have no conflicts of interest to disclose.

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NAFLD and Cardiovascular and Cardiac Disease: Clinical Implications

9

Eleonora Scorletti and Christopher D. Byrne

9.1 Introduction

Cardiovascular disease (CVD) is an umbrella term used to describe a cluster of disorders of heart and blood vessels, and include among others: hypertension, coronary heart disease, arrhythmias, cerebrovascular disease, peripheral vascular disease, heart failure and cardiomyopathies. Despite a marked reduction in the rate of age-standardised CVD death over the past 30 years, the burden of CVD remains high [1, 2]. According to the WHO, CVD is the most common cause of death in the Westernised countries (35% of all deaths) and by 2030, almost 23.6 million people will die from CVD, mainly from heart disease and stroke. Due to the high morbidity, mortality and healthcare costs associated with CVD, it is crucial to investigate the effects of non-alcoholic fatty liver disease (NAFLD) on the development of cardiovascular events in order to organise an efficient health prevention and treatment programme to identify the risk of developing CVD in patients with NAFLD. Currently, it is difficult to prove an independent role for NAFLD in the development of CVD

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as this liver condition is often embedded in a more complex metabolic syndrome involving insulin resistance, dyslipidaemia, central adipose tissue dysfunction and gut microbiota alteration. In this chapter, we aim to explain some of the liver-centred mechanism associated with CVD that may explain why NAFLD is a risk factor for CVD. We describe the role of: (1) hepatic 'selective insulin resistance' with consequent reduction of nitric oxide production leading to endothelial dysfunction; (2) hepatic structural changes and the development of non-cirrhotic portal hypertension associated with left ventricular dysfunction; (3) increases in de novo lipogenesis and its association with atherogenic dyslipidaemia; (4) liver hepatokines which are associated with CVD; (5) coagulation factors that have a role in the thrombotic process and (6) PNPLA3 I148M genotype and its association with ischaemic heart disease.

9.2 Epidemiology

In the past few decades, there has been a decline in age-standardised CVD mortality rates worldwide [3]. From 1990 to 2013, the annual age-adjusted cardiovascular mortality rates have declined, falling by 22% in nearly all regions of the world, especially in high-income North America, Western Europe, Japan, Australia and New Zealand [1, 3–5]. The age-standardised rates of death due to CVD fell 15.6%, whereas, global CVD deaths rose by 12.5% between 2005 and 2015. These age-standardised rates of death reductions were largely driven by declining mortality rates due to cerebrovascular disease (i.e. stroke; decreased by 21.0%) since 2005 [6]. Most of the epidemiological studies on CVD morbidity and mortality used the IMPACT Coronary Heart Disease Model that is a statistical model employed to examine the relative contributions of medical and surgical interventions for coronary heart disease versus preventive strategies that target the reduction of major coronary heart disease risk factors [1, 7–9]. Using this model, Ford et al. were able to estimate that approximately 47% of the decline in coronary heart disease mortality rate was attributable to changes in medical and surgical treatments including secondary preventive therapies. Whereas, risk-factor changes accounted for approximately 44% of the decrease in deaths and was attributed to primary prevention with changes in risk factors, including reductions in total cholesterol (24%), systolic blood pressure (20%), smoking prevalence (12%) and physical inactivity (5%) [10]. In another study on Swedish population, Björck et al. reported that 75% of the mortality reduction came from primary prevention and that the major contributors to the mortality reduction were dietary changes [11].

However, despite a decline in CVD mortality in the first half of twentieth century, the increase in prevalence of obesity, metabolic syndrome, NAFLD, and type 2 diabetes is likely to be responsible for a slowing in the decline of CVD mortality rates. Ford et al. showed that the increased prevalence in BMI and type 2 diabetes accounted for an increase in CVD mortality of 8% and 10%, respectively [10]. A recent meta-analysis conducted by Targher et al. investigated the association between NAFLD and risk of incident CVD [12]. The presence of NAFLD

was associated with an increased risk of a fatal and non-fatal CVD events such as myocardial infarction, angina, stroke, or coronary revascularisation [12]. Based on 16 observational prospective and retrospective studies comprising 34,043 adult individuals (36.3% with NAFLD), patients with NAFLD were found to have a higher risk of fatal and/or non-fatal CVD events considered together (random effect OR 1.63, 95% CI 1.06–2.48, I^2 = 83%; p = 0.02) than those without NAFLD. Additionally, presence of more severe NAFLD with fibrosis was associated with an increased risk of CVD mortality (random effect OR 3.28, 95% CI 2.26–4.77, I^2 = 0) as well as an increased risk of fatal and non-fatal CVD events considered together (random effect OR 1.94, 95% CI 1.17–3.21, I^2 = 23%) [12]. In a recent cross-sectional study in South Korean population, Lee et al. investigated the influence of NAFLD on subclinical coronary atherosclerosis detected by coronary computed tomography angiography in an asymptomatic population. This study showed that patients with NAFLD (diagnosed by ultrasound) had a higher coronary calcium score than those without NAFLD ($p < 0.001$) [13]. In addition, the odds ratios adjusted for cardiovascular risk factors (age, sex, obesity, diabetes mellitus, hypertension, hyperlipidaemia, current smoking, family history of CAD and hs-CRP) for any atherosclerotic plaque was 1.18; 95% CI 1.03–1.35; p = 0.016 and for non-calcified plaque was 1.27; 95% CI 1.08–1.48; p = 0.003 with NAFLD [13]. This is the largest study to date to describe the association between NAFLD and atherosclerotic plaque. In a retrospective single-centre study, Pais et al. presented a cross-sectional and longitudinal evidence that NAFLD is an important risk factor for the development of early carotid atherosclerosis [14]. The authors examined the impact of steatosis (diagnosed with the fatty liver index – FLI[1] [15]) on the presence and progression of carotid intima-media thickness and carotid plaques. They found that steatosis independently predicted carotid intima-media thickness (p = 0.002) after adjustment for metabolic syndrome and cardiovascular risk factors. Steatosis at baseline predicted carotid plaque occurrence (OR = 1.63, 95% CI 1.10–2.41, p = 0.014), independently of age, sex, type-2 diabetes, tobacco use, C-reactive protein, hypertension, and carotid intima-media thickness. Interestingly, in a post-hoc analysis of a prospective Japanese cohort study where NAFLD was diagnosed by ultrasound the adjusted hazard ratios for incident CVD were 10.4 (95% confidence interval 2.61–44.0, P = 0.001) in non-overweight with NAFLD, 1.96 (0.54–7.88, P = 0.31) in overweight without NAFLD and 3.14 (0.84–13.2, P = 0.09) in overweight with NAFLD [16]. However, there was a 12-year gap between the enrolment and the post-hoc analysis without an update on more recent information leading to possible bias in the analysis. In addition, the diagnosis of CVD was made by self-administered questionnaire and there was no information on dietary habits and genetic polymorphisms. Nevertheless, this study showed a potential role of NAFLD on CVD not associated with obesity. Thus, the majority of current evidence suggest that there is an independent association between NAFLD and CVD.

[1] FLI: $= (e^{0.953 \times \text{loge(triglycerides)} + 0.139 \times \text{BMI} + 0.718 \times \text{loge(GGT)} + 0.053 \times \text{waist circumference} - 15.745}) / (1 + e^{0.953 \times \text{loge(triglycerides)} + 0.139 \times \text{BMI} + 0.718 \times \text{loge(GGT)} + 0.053 \times \text{waist circumference} - 15.745}) \times 100$

9.3 Aetiology and Pathogenesis

The liver is anatomically linked to the cardiovascular system through the hepatic veins which drain blood into the inferior vena cava. In the presence of lipid accumulation in the hepatocytes, the liver undergoes structural changes depending on the degree of severity of liver disease and the presence of fibrosis or ballooning. These changes affect the structure not only of the hepatocytes that become swollen due to lipid accumulation and inflammation (ballooning), but there is also a change in the structure of hepatic sinusoids, bile ducts, hepatic arterioles and the space of Disse [17]. These structural changes along with the liver dysfunction with the production of hepatokines and dysregulation of glucose and lipid metabolism might contribute to the pathogenesis of CVD.

9.3.1 Selective Insulin Resistance and Structural Changes in the Liver

Endothelial dysfunction is the primary cause of vascular dysfunction, and it is one of the earliest markers of atherosclerosis. Recent studies showed that endothelial dysfunction, which is potentially responsible for CVD development, and increased risk of incident hypertension were associated with NAFLD [18–24]. The mechanism underlying the correlation between NAFLD and endothelial dysfunction is not completely understood. One possible mechanism associated with endothelial dysfunction in patients with NAFLD could be the presence of 'selective hepatic insulin resistance', affecting both the liver and the vasculature. With insulin resistance there are two effects: (a) insulin fails to suppress gluconeogenesis as well as lipogenesis and (b) there is an impaired production of nitric oxide leading to endothelial dysfunction [25] (see Fig. 9.1a,b). The liver expresses both insulin receptors IRS1 and IRS2. IRS2 expression is regulated by insulin levels in fasting and post-meal state, whereas IRS1 expression is not affected by insulin and therefore remains unaltered in both fasting state and immediately after food intake. The required condition for the development of 'selective insulin resistance' is the presence of an altered ratio between IRS1 and IRS2 with a reduced expression of IRS2 and increase expression of IRS1. Research studies show that increased liver fat is associated with both increased expression of IRS1 and impaired insulin clearance contributing to the development of hepatic insulin resistance [26]. In the physiological state, insulin is involved in cardiac metabolism, promoting glucose uptake, protein synthesis, regulation of long-chain fatty acid metabolism, and vascular tonicity. Moreover, insulin has opposing haemodynamic actions on blood vessels as it regulates the endothelial vasoconstriction and vasodilation in two ways: (1) via the phosphorylation of the IRS2 and activation of phosphatidyl inositol 3-kinase (PI3K)/Akt pathway, responsible for the nitric oxide production [27]; and (2) via phosphorylation of the IRS1 the activation of mitogen-activated protein kinase (MAPK) pathway, regulating the secretion of endothelin-1 [25], see Fig. 9.1a. Therefore, insulin regulates the balance between nitric oxide-mediated vasodilation

and endothelin-1-mediated vasoconstriction. In the first pathway, the activation of (PI3K)/Akt in endothelial cells leads to phosphorylation of endothelial nitric oxide synthase that in turn synthesises nitric oxide from the guanidine group of arginine (L-arginine) and O_2. This pathway regulates the expression of nitric oxide synthase, vascular endothelial growth factor, antioxidant haeme oxygenase-1, and vascular cell adhesion molecule-1. The action of nitric oxide on the endothelium is primarily mediated via reductions in intracellular calcium concentrations promoting vasodilation. In the second pathway, the activation of Grb2 and Shc causes a cascade of

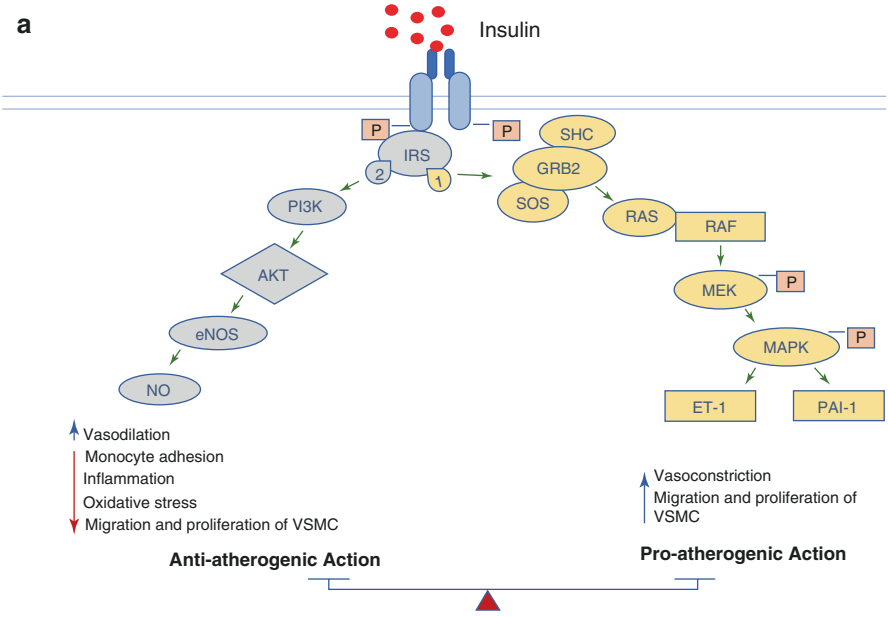

Fig. 9.1 Insulin signalling in endothelial cells. (**a**) Insulin has opposing haemodynamic actions in blood vessels. (1) Pro-atherogenic action: Insulin regulates endothelial vasoconstriction via phosphorylation of insulin receptor 1 (IRS1) and the activation of mitogen-activated protein kinase (MAPK) pathway, regulating the secretion of endothelin-1 (ET-1) and plasminogen activator inhibitor-1 (PAI). (2) Anti-atherogenic action: insulin affects vasodilation via the phosphorylation of insulin receptor 2 (IRS2) and activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway, responsible for nitric oxide production (NO). (**b**) Selective insulin resistance. Reduced expression of IRS2 leads to a selective inhibition of the (PI3K)/Akt pathway causing a deterioration of intracellular signalling that reduces NO synthesis. High extracellular concentration of glucose increases the synthesis of superoxide (O^-_2) dependent of NAD(P)H oxidase, which reacts with NO to generate peroxynitrite ($ONOO^-$), contributing to endothelial cell dysfunction. IRS1 expression is unchanged or increased therefore the (MAPK) pathway is not inhibited resulting in enhanced expression of endothelin-1 and proliferation of vascular smooth muscle cells with pro-atherosclerotic action. In addition, impaired insulin signalling causes a reduction in outward potassium (K^+) currents causing abnormal repolarisation in cardiomyocytes. *IRS1* and *IRS2* insulin receptors 1 and 2, *MAPK* mitogen-activated protein kinase, *ONOO*$^-$ peroxynitrite, *NO* nitric oxide, *PI3K* phosphatidylinositol-4,5-bisphosphate 3-kinase; nitric oxide synthase, *O*$^-_2$ superoxide, *K*$^+$ potassium, *ET-1* endothelin-1, *PAI* plasminogen activator inhibitor-1

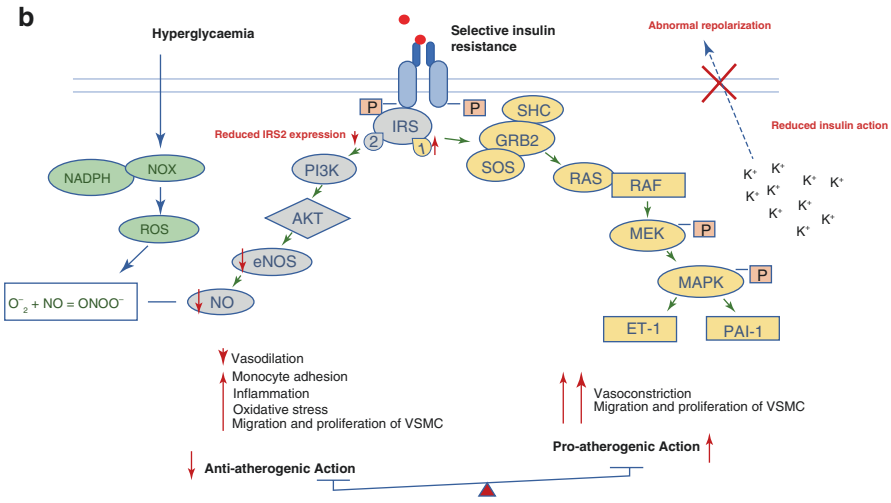

Fig. 9.1 (continued)

phosphorylation to activate the MAPK pathway with subsequent secretion of endothelin-1 [25]. Endothelin-1 plays an important role in vascular function through its action on vascular smooth muscle cells, oxidative stress proliferation and apoptosis [28, 29]. Reduced nitric oxide production and/or bioavailability are associated with hypertension, atherosclerosis and angiogenesis-associated disorders. A recent study from Persico et al. showed that nitric oxide synthase phosphorylation was reduced in liver samples obtained from both NASH and NAFLD patients, compared to liver samples from healthy control subjects [30]. These authors also found that endothelial dysfunction measured with flow-mediated dilation of the brachial artery was reduced according to liver disease severity. In the absence of nitric oxide signalling, there is a disturbance in vascular homeostasis, triggering a series of events leading to pathologies such as hypertension, renal vascular insufficiency and chronic heart failure [31, 32], see Fig. 9.1b.

In the presence of endothelial insulin resistance, the (PI3K)/Akt pathway and (MAPK) pathway are selectively impaired resulting in a ‘selective insulin resistance’ state. In this state, there is a selective inhibition of the (PI3K)/Akt pathway causing a deterioration of intracellular signalling that reduces the L-arginine transport with consequent reduction of NO synthesis. By contrast the (MAPK) pathway is not inhibited [33] resulting in enhanced expression of endothelin-1 and proliferation of vascular smooth muscle cells with pro-atherosclerotic action [34, 35]. Multiple pathophysiological stimuli typical of NAFLD such as increased production of inflammatory cytokines, hyperglycaemia, high levels of asymmetric dimethylarginine [36], hypoadiponectinemia [37] and increased release of free fatty acids can also cause a selective inhibition of the (PI3K)/Akt pathway with consequent reduction of NO production. In addition, high extracellular concentrations of D-glucose

increase synthesis of O^{-}_{2} dependent of NAD(P)H oxidase, which reacts with the NO to generate $ONOO^{-}$, contributing to endothelial dysfunction [32], see Fig. 9.1b.

Insulin has also a direct effect on cardiomyocytes, modulating cardiac contractility and affecting cardiac output. Moreover, insulin mediates the cellular hypertrophy and generates an antiapoptotic effect on cardiomyocytes by activating other intermediary intracellular signalling pathways that affect potassium currents [38, 39]. Impaired insulin signalling causes a reduction in the outward K+ currents causing abnormal repolarisation in cardiomyocytes [40]. The arrhythmogenic potential of altered outward K+ currents can contribute to an increase in the incidence of heart failure [41]. Several studies have assessed the association between NAFLD and left ventricular dysfunction and hypertrophy [42]. In a multicentre community-based Coronary Artery Risk Development in Young Adults (CARDIA) study, VanWagner et al. have performed a cross-sectional analysis of 2713 participants with imaging-diagnosed NAFLD. Theses authors showed that NAFLD was independently associated with left ventricular systolic and diastolic dysfunction and myocardial remodelling [43]. In a recent cross-sectional study during a health screening programme, 3300 subjects underwent echocardiography and hepatic ultrasonography. In this study, the presence of NAFLD was independently associated with a 68% increase in the risk of left ventricular diastolic dysfunction. After adjusting for age, sex and waist circumference, the risk of diastolic dysfunction incrementally increased according to the severity of fibrosis. After stratifying the population according to BMI, the association between NAFLD with fibrosis and LV diastolic dysfunction was significant only in non-obese subjects [21].

Steatosis, inflammation and fibrosis cause significant structural changes in the liver that might explain the endothelial and myocardial dysfunction described in this metabolic liver condition. Recent evidence showed that hepatic parenchymal alterations are responsible for the biomechanical and rheological changes in patients with NAFLD [17]. Hepatocyte enlargement due to hepatocellular lipid accumulation and ballooning may cause changes in the hepatic microvasculature [44] with sinusoidal compression, sinusoidal space restriction, distortion of the sinusoidal pattern (reducing the sinusoidal space by as much as 50% compared with normal liver) [45], compression of sinusoids and loss of fenestrae resulting in impaired sinusoidal flow with increase in intrahepatic resistance causing an increase in portal venous pressure [46, 47]. In this condition, there is a disruption of sinusoidal flow starting in zone 3 of the liver (from the central vein) and then expanding in through the entire lobule. With these structural and functional changes, liver sinusoidal endothelial cells become defenestrated and deposit extracellular matrix within the space of Disse causing relative hypoxia [45]. Experimental studies in steatotic animal models indicate that moderate steatosis reduces sinusoidal blood flow by approximately half because of distortion of the sinusoids by fat-filled hepatocytes [48]. These alterations are associated with increase in intrahepatic resistance responsible of post-sinusoidal non-cirrhotic portal hypertension [49]. Franque et al. studied the portal pressure in 50 patients with non-alcoholic fatty liver disease using transjugular liver–vein catheterisation and biopsy. They found that the hepatic venous pressure gradient was ≥5 mmHg; the threshold indicating sinusoidal portal hypertension in about

one-third of the study population, and that this portal hypertension was related to the steatosis grade and not to the presence of extensive fibrosis or cirrhosis [50]. In another study, Chung et al. showed that NAFLD was associated with a 29% increase in the risk of diastolic dysfunction compared with controls. In addition, the authors found that in non-obese subjects, the risk to develop diastolic dysfunction increased incrementally according to fibrosis grade [21].

These hepatic haemodynamic changes in patients with NAFLD suggest that there could be a reduction in hepatic arterial flow [51] with a consequent decrease in cardiac preload resulting in early asymptomatic cardiovascular alterations [52]. This would have an effect on the micro and macro circulation with possible increase in vascular calcifications and atherosclerosis formation [53], endothelial dysfunction [30] and increase in intima-media thickness [54] and myocardial dysfunction [42, 55, 56].

9.3.2 Lipid Metabolism and Atherosclerosis

Several studies have shown that the process of atherogenesis is initiated by two main mechanisms: (1) endothelial injury and/or (2) accumulation of low-density lipoproteins (LDL) within the arterial wall, which are generally prone to oxidisation [57, 58].

The liver plays a major role in regulating lipid metabolism by the combined action of de novo lipogenesis and lipid oxidation, as well as uptake and secretion of lipoproteins. Liver fat accumulation is associated with an imbalance in hepatic fatty acid uptake, endogenous lipid synthesis, lipid oxidation and very-low-density lipoprotein production [59, 60]. NAFLD is associated with hepatic insulin resistance and induces hepatic VLDL production via changes in the rate of apo B synthesis [61] and stimulation of de novo lipogenesis [62]. In the presence of hepatic insulin resistance, there is an increased expression of sterol regulatory element binding protein 1c (SREBP1c) that leads to the activation of key enzymes for de novo lipogenesis [63]. Moreover, carbohydrate responsive-element binding protein (ChREBP) is also stimulated by hyperglycaemia contributing to the activation of lipogenesis [64].

In the liver, lipid droplets are stored in the endoplasmic reticulum of the hepatocytes where VLDL particles are assembled. Subsequently, apolipoprotein B-containing VLDL particles are secreted into the circulation. Increased circulating levels of VLDL particles can lead to the generation of small, dense LDL that are highly atherogenic. In the circulation, LDL can enter the artery wall and be oxidised by vascular cells (endothelial cells, smooth muscle cells and macrophages) with oxidising enzymes including lipoxygenase and myeloperoxidase. Oxidation of LDL can occur in two ways: (a) mild oxidation of LDL, with absence of changes or little changes in apolipoprotein B100 (this mild oxidised LDL retains its affinity for the LDL receptor); (b) mild oxidised LDL can be further oxidised leading to a loss of recognition by the LDL receptor and a shift to recognition by scavenger receptors [58]. Oxidised LDL activates the conversion of monocytes to macrophages foam cells with subsequent formation of the fatty streak. In addition, the

reduction of NO bioavailability (described previously) with consequent increase in the production of reactive oxygen species such as O^{-}_{2} and $ONOO^{-}$ contribute to the oxidative modification of LDL and the development of atherosclerosis. The accumulation of subendothelial atherogenic apolipoprotein B-containing low-density and very-low-density lipoproteins and chylomicrons plus monocytes activation and migration through the endothelial wall into the vascular smooth muscle cells layer of the intimal media contribute to the formation of the atherogenic streak. Several studies have showed an association between NASH and an altered LDL profile [59, 60]. Chalasani et al. showed a significant association between NASH and increased levels of oxidised LDL compared with controls. This was in line with other studies conducted previously by Sanyal and MacDonald where they found an association between lipid peroxidation and severity of liver disease. Alkhouri et al. showed that in patients with NAFLD, the histologic severity of liver disease was strongly associated with an increased level of triglycerides and low-density lipoprotein and a decrease in high-density lipoprotein [65]. In addition, in a large, multi-ethnic, sex-balanced cohort, CT-diagnosed NAFLD was associated with atherogenic dyslipidaemia defined as low HDL-cholesterol and high triglycerides and a triglycerides/HDL ratio greater than 3 [60].

9.3.3 Hepatokines

The ectopic accumulation of lipids in the liver is associated with the infiltration and activation of immune cells and production of pro-atherogenic and pro-inflammatory cytokines known as hepatokines. Hepatokines are proteins that influence metabolism and inflammatory pathways by affecting insulin sensitivity, homeostasis and cardiovascular health [66]. The liver secretes numerous hepatokines; however, the specific role of these hepatokines is not been completely elucidated. Some of them have been associated with NAFLD and CVD although the exact role has not been clarified. Fetuin-A (also known as α2-HS-glycoprotein), that is primarily synthesised by hepatocytes, is a natural inhibitor of the insulin receptor tyrosine kinase. Several lines of evidence showed that fetuin-A is a potent inhibitor of calcification. Fetuin-A binds with bioactive Ca^{2+} suggesting its potential role in the inhibition of systemic calcification by protecting VSMC from the detrimental effects of Ca^{2+} overload and subsequent calcification [67, 68]. However, the role of fetuin-A in NAFLD and CVD seems to be complex and controversial as it seems to be modulated by various independent pathogenetic mechanisms such as inflammation and insulin resistance. Sato et al. showed that serum fetuin-A concentration was negatively correlated with platelet count, NAFLD fibrosis score and mean IMT [69]. In contrast, Celebi et al. observed no difference in plasma levels of fetuin-A between NASH and NAFLD groups. Moreover, the authors did not find any association of circulating fetuin-A with liver histology and insulin resistance in subjects with NAFLD [70]. By contrast, some studies showed high plasma levels of fetuin-A with insulin resistance and hepatic steatosis and increased risk of myocardial infarction and ischemic stroke [71]. Kahraman et al. described high plasma concentrations of

fetuin-A in patients with NASH; this result was confirmed by mRNA and protein expression of fetuin-A in liver tissue [72]. Fetuin-A could represent a possible biomarker to detect CVD in patients with NAFLD; however, further studies are needed to clarify its metabolic function and its association with liver disease, atherosclerosis and vascular calcification.

Fibroblast growth factor 21 is another hepatokine secreted mainly by the liver and is regulated by several transcription factors including peroxisome proliferator-activated receptor α (PPAR- α), PPARγ, ChREBP and SREBP [73]. Fibroblast growth factor 21 has been shown to have beneficial effects on energy homeostasis, glucose and lipid metabolism. Emerging evidence suggests that fibroblast growth factor 21 is also a physiological protector of vascular functions via two major mechanisms: (1) indirectly via inducing expression and secretion of adiponectin that in turn leads to the production of NO in endothelial cells [74, 75]; and (2) directly by inhibiting the hepatic cholesterol biosynthesis by suppressing SREBP [76]. However, in contrast to this evidence, recent studies showed that high levels of fibroblast growth factor 21 are associated with NAFLD and atherosclerosis [77, 78].

Selenoprotein P is a secretory protein primarily produced and released by the liver, and it is responsible for transporting selenium from the liver to extrahepatic tissues. Selenoprotein P is upregulated in the liver of patients with NAFLD [79], type 2 diabetes [80] and CVD [81]; however, there have been very few studies that have investigated the relationship between selenoprotein P and CVD. The mechanism by which selenoprotein P causes CVD is not clear; one possible mechanism is through its effect on insulin resistance. However, further studies are needed to identify the independent relationship between selenoprotein P and CVD and to clarify the underlying mechanism linking selenoprotein P and CVD.

Hepatokines that are mainly secreted from the liver have para- and endocrine effects and are known to directly affect inflammation, and glucose and lipid metabolism. Although accumulating evidence shows that hepatokines play an important role in modulating inflammatory processes that in turn affect atherosclerotic process, it remains controversial whether there is an independent effect of these hepatokines to affect the pathogenesis of CVD.

9.3.4 Prothrombotic Factors

The liver synthesises several coagulation factors including fibrinogen, and plasminogen activator inhibitor-1 (PAI-1), which may have important roles in the development of CVD. In addition, insulin has also been shown to increase the expressions of PAI-1, through the MAPK pathways [33]. In a large community-based, prospective observational study of CVD risk, increasing PAI-1 levels were associated with an adverse cardiovascular risk profile [82].

Kotronen et al. showed an independent association between increased activities of coagulation factors (FVIII, FIX, FXI and FXII) and NAFLD (liver fat diagnosed by MRS) compared with controls [83]. This study was in accordance with Tripodi et al. showing that plasma from patients with NAFLD was characterised by

a procoagulant imbalance that progressed with increasing severity of disease from simple steatosis to cirrhosis [84].

By contrast, Verrijken et al. studied a large cohort of overweight/obese patients who underwent a clinical assessment for coagulation factors, and metabolic and liver disease. In this study, severity of liver histology was associated with a significant and independent increase in PAI-1. Whereas, other metabolic features (but not NAFLD) were associated with an increase in fibrinogen, factor VIII and von Willebrand factor, antithrombin III was decreased [85]. Similar results were found in other research studies in adults and children where increased PAI-1 levels were associated with NAFLD severity and CVD [86–88]. PAI-1 is the primary inhibitor of the endogenous fibrinolytic system, and it is responsible for reducing fibrinolytic activity and plays a key role in the atherothrombotic process [86, 89, 90]. Increased PAI-1 plasma levels would reduce the capacity of the fibrinolytic system to prevent fibrin deposition in vessel walls and thrombus formation [91].

9.3.5 PNPLA3 I148M Genotype

The relationship between liver fat content, NAFLD and ischaemic heart disease (IHD) has recently been investigated in a Mendelian randomisation and meta-analysis of 279,013 individuals [92]. In a cohort study of the Danish general population (n = 94,708/IHD = 10,897), the authors tested whether a high liver fat content or a diagnosis of NAFLD was associated with IHD. The authors then tested whether a genetic variant in the gene encoding the protein patatin-like phospholipase domain containing 3 proteins (PNPLA3), I148M (rs738409) (a strong and specific cause of high liver fat content and NAFLD) was causally associated with the risk of IHD.

As expected from existing evidence, the authors found that the risk of IHD increased stepwise with increasing liver fat content (in quartiles) up to an odds ratio (OR) of 2.41 (1.28–4.51) (P-trend = 0.004). The corresponding OR for IHD in individuals with vs. without NAFLD was 1.65 (1.34–2.04) ($P = 3 \times 10^{-6}$), which is in keeping with existing evidence. PNPLA3 I148M was associated with a stepwise increase in liver fat content of up to 28% in MM vs. II-homozygotes (P-trend = 0.0001) and with ORs of 2.03 (1.52–2.70) for NAFLD ($P = 3 \times 10^{-7}$), 3.28 (2.37–4.54) for cirrhosis ($P = 4 \times 10^{-12}$) and 0.95 (0.86–1.04) for IHD (P = 0.46). In the meta-analysis (N = 279,013/IHD = 71,698), the OR for IHD was 0.98 (0.96–1.00) per M-allele vs. I-allele. The OR for IHD per M-allele for higher genetically determined liver fat content was 0.98 (0.94–1.03) vs. an observational estimate of 1.05 (1.02–1.09) (P for comparison = 0.02).

Therefore, despite confirming the known observational association of hepatic fat content (and NAFLD) with the risk of prevalent IHD in this analysis, the authors suggested that fatty liver due to PNPLA3 variant is not causally associated with IHD [92]. Although, these data are undoubtedly thought provoking, we believe that it is important to be cautious about the interpretation of these data for the following reasons. (a) Based on the ICD-8 codes (and computed tomography scanning), the prevalence of NAFLD (i.e. 0.7% of the whole cohort; 633 out of 94,708) was

extraordinarily low, and it is also quite possible that there was contamination bias (with up to 25–30% of subjects in the reference group possibly having undiagnosed NAFLD). (b) Using a Mendelian randomisation approach, the authors failed to show any increase in the risk of prevalent IHD with the presence of the PNPLA3 148M allele in a subgroup of 1439 individuals in whom liver fat content was detected by computed tomography scanning. It is important to note that many subjects in this analysis did not have NAFLD (because liver fat percentage was <5.6%), and it is also noteworthy that the mean liver fat percentage was extremely low and similar in all three PNPLA3 genotypes (II = 5.1%, IM = 6.0% and MM = 6.5%, respectively). (c) The authors also tested whether the PNPLA3 genotype was associated with risk of prevalent IHD in the whole cohort, of whom nearly 99% did not have known NAFLD. Since PNPLA3 148 MM was associated with a tiny increase in liver fat percentage in people with imaging-diagnosed NAFLD, it is perhaps not surprising that in the general population without NAFLD, PNPLA3 148 MM was not associated with IHD. Although a subsequent meta-analysis also confirmed the lack of a significant association between this genetic variant and IHD, again no information was available about NAFLD status in the CARDIOGRAMplusC4D consortium.

To date, a consensus is emerging that there are at least two distinct forms of NAFLD, i.e. the obese/metabolic NAFLD and the PNPLA3-associated NAFLD, which may have different consequences for risk of IHD [12, 93–95]. Less than 5–6% of European individuals with NAFLD carry the PNPLA3 148MM genotype, and this genotype is neither sufficient nor necessary to cause non-alcoholic steatohepatitis, cirrhosis or primary liver cancer. The contribution of genetic polymorphisms to inter-individual variation in NAFLD phenotype is relatively small, and the role of the PNPLA3 148M allele in the general population without NAFLD is far from clear.

Thus, we consider that further research is urgently needed to test the effect of PNPLA3 148 MM genotype on risk of incident cardiovascular outcomes in cohorts with proven NAFLD. Since undiagnosed NAFLD is very common in the general ‘healthy’ population, it is also important to know that the control/reference population does not have NAFLD.

9.4 Treatments

Since NAFLD is associated with extrahepatic complications such as type 2 diabetes (T2DM) and chronic kidney disease that also increase risk of cardiovascular disease (CVD) [12, 96–98], effective treatment strategies are urgently required [94]. Crucially, similar proportions of people with NAFLD die from CVD as from liver disease [94] and when patients with NAFLD develop type 2 diabetes, the presence of diabetes further increases risk of CVD, creating a vicious spiral of potential ill health [99]. Consequently, an ideal effective treatment for NAFLD might therefore be expected to not only reduce risk of chronic liver disease-related complications but also to decrease risk of type 2 diabetes and CVD.

In 2017, the comparative benefits and harms of different interventions using standard Cochrane methodology were evaluated [100]. These authors concluded that due to the very low-quality evidence, there was current uncertainty about the effectiveness of pharmacological treatments for people with NAFLD including those with NASH. Importantly as stated, further well-designed randomised clinical trials with sufficiently large sample sizes are necessary. Nevertheless, that said, the purpose and focus of this section are to discuss the existing evidence for potential diets and pharmacological treatments for NAFLD which also have beneficial effects on CVD and CVD risk factors.

The ability to diagnose NASH and monitor NASH is crucial for the testing of therapeutic interventions for NASH and to evaluate their effectiveness on CVD and cardiac complications of NAFLD.

Currently, the only investigation with acceptable sensitivity and specificity for diagnosing and monitoring NASH is liver biopsy and histological examination; and this is the current 'gold standard' that has undoubtedly hampered the testing of drug effectiveness in NASH [101]. Despite this caveat, current guidelines have concluded that there is sufficient evidence to consider the use of a Mediterranean diet (MD) [102] and pioglitazone or vitamin E therapy in the treatment of NASH [102–104].

Weight loss is the most effective way to promote liver fat removal, and several controlled studies have confirmed that an intense approach to lifestyle changes, carried on along the lines of cognitive-behaviour treatment, is able to attain the desired 7–10% weight loss, associated with reduced liver fat, non-alcoholic steatohepatitis (NASH) remission and also reduction of fibrosis [105]. Even larger benefits have been reported after bariatric surgery in NAFLD, where 80% of subjects achieve NASH resolution at 1-year follow-up [105].

The major focus of this section will be to discuss the potential CVD benefits of the MD diet as well as pioglitazone and vitamin E as this diet and these two agents have recently been recommended by the Guidelines discussed above for NAFLD. We will also discuss the role of statins as these agents have been used for many years to lower low-density lipoprotein (LDL-C) and decrease CVD risk.

9.4.1 Mediterranean Diet (MD)

The benefits of the MD as the diet of choice for NAFLD have recently been discussed in an excellent review of the subject [106]. The individual components of the MD such as olive oil, fish, nuts, whole grains, fruits and vegetables have been shown to beneficially affect or negatively correlate with NAFLD. Additionally, an MD contains lower amounts of dietary components that are thought to be potentially harmful for obesity, NAFLD and CVD, such as fructose, refined carbohydrates, trans fatty acids and red meats and therefore an MD diet tends to comply with current guidelines to reduce the risk of CVD [107]. In June 2017, the American Heart Association's presidential advisory on dietary fats stated that replacing saturated fat with polyunsaturated vegetable oil reduces the incidence of CVD by ~30% [108].

Importantly, this shift towards more unsaturated fats occurs when a Westernised diet containing processed foods is replaced by the Mediterranean diet (MD) [108]. It is beyond the scope of this chapter to discuss the many potential mechanisms of benefit by which a MD may benefit NAFLD and CVD. However, we have briefly summarised the key components of the diet and the key factors that may be favourably affected in reducing risk of CVD in NAFLD in Fig. 9.2.

Data from three small, brief duration randomised trials have suggested a potential beneficial effect of the MD in NAFLD [109–111]. We believe that longer-term RCTs are needed, preferably with histological liver outcomes to test whether there is any benefit on NASH and/or liver fibrosis. It has to be stressed that in most cases any form of healthy diet, which leads to caloric reduction and is acceptable to the patient, should be encouraged for patients with NAFLD. For the patient who finds caloric restriction difficult, changing dietary composition without necessarily reducing caloric intake could offer a more feasible alternative although the benefit on liver health is not as marked as weight reduction alone [102, 105]. The importance of weight loss has been highlighted in patients with NASH, where weight loss per se is able to induce NASH resolution, without any worsening of fibrosis [112].

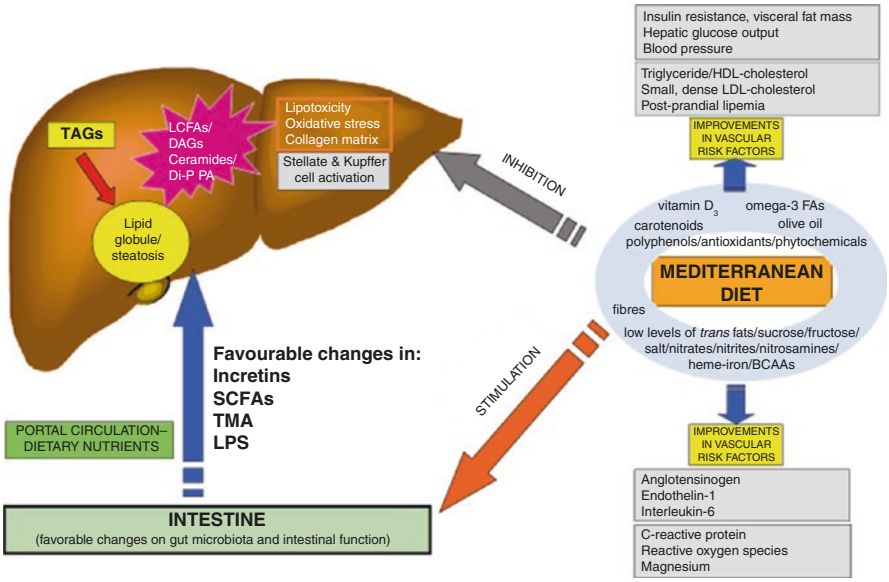

Fig. 9.2 Potentially beneficial effects of the Mediterranean diet in NAFLD. The Mediterranean diet (MD) contains a variety of nutrients that have the potential for affecting improvements in vascular risk factors. The MD may have stimulatory effects in the intestine to promote favourable changes in gut microbiota and intestinal function with beneficial effects for liver disease in NAFLD. *BCAAs* branched chain amino acids, *DAGs* di-acylglycerols, *di-P PA* di-palmitolyl phosphatidic acid, *LCFAs* long-chain fatty acids, *LPS* lipopolysaccharide, *SCFAs* short chain fatty acids, *TAGs* tri-acylglycerols, *TMA* trimethylamine

9.4.2 Thiazolidienediones (Pioglitazone) and Vitamin E

Recently as a result of several randomised placebo-controlled trials in patients with NASH, three key international bodies (National Institute for Health and Care Excellence (NICE), the Joint European Societies (Diabetes, Hepatology and Obesity) and the American Association for the Study of Liver Diseases) have recently also recommended the use of pioglitazone for the treatment of NASH [102–104].

9.4.2.1 Pioglitazone

Thiazolidienediones (TZDs) are well known to lower plasma glucose concentrations over many years of treatment, and these drugs (rosiglitazone and pioglitazone) have been licensed for the treatment of type 2 diabetes for almost 20 years. TZDs are potent peroxisome proliferator-activated receptor gamma (PPARγ) agonists that target both adipose tissue metabolism and also inflammation. The first available TZD was troglitazone which was rapidly withdrawn (in the UK in 1997, and in the USA in 2000) because of toxic side effects. In the UK rosiglitazone was withdrawn as a result predominantly of concerns raised about possible increased cardiovascular risk in a meta-analysis published in 2007 [113]. Yet despite those concerns, in 2013 the US Food and Drug Administration (FDA) lifted the final regulatory restrictions on rosiglitazone in 2013, stating that 'we have continued monitoring these medicines and identified no new pertinent safety information. As a result, we have determined the risk evaluation and mitigation strategy is no longer necessary to ensure that the benefits of rosiglitazone medicines outweigh their risks'. Since most of the available evidence with this class of drug in NASH exists for pioglitazone, because of these problems with rosiglitazone, we will focus discussion on the evidence with pioglitazone treatment.

Pioglitazone treatment results in histological resolution of NASH in ~50% of patients regardless of diabetes status [114–116]. The mean effect for response to pioglitazone defined as resolution of NASH from three key trials [114–116] is 51% (95% CI 42, 60), and a recent meta-analysis of pioglitazone treatment in NASH has concluded that thiazolidinediones significantly improve ballooning degeneration, lobular inflammation, steatosis and combined necroinflammation in patients with NASH and that pioglitazone may improve fibrosis [117].

Extensive use of pioglitazone to treat T2DM has established its safety and generic pioglitazone costs to the UK NHS are only ~£1.15 (1.31 Euros or 1.51 USD in June 2018); per patient per month. Importantly since patients with NAFLD are also at increased risk of type 2 diabetes and CVD, RCT evidence also shows that treatment with pioglitazone also decreases risk of developing type 2 diabetes [118], myocardial infarction [119] and stroke [120, 121]. For all of these additional benefits of treatment with pioglitazone, the magnitude of the benefit of treatment with pioglitazone is a reduction in risk of between 16 and 72%. For example in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events) [119], the main secondary end point was the composite of all-cause mortality, non-fatal myocardial infarction and stroke. 301 patients in the pioglitazone group and 358 in the placebo group reached this end point, and there was

a significant 16% decrease in risk of this end point with pioglitazone treatment. In order to examine whether pioglitazone can reduce the risk of type 2 diabetes mellitus in adults with impaired glucose tolerance, a total of 602 patients were randomly assigned to receive pioglitazone or placebo. After a median follow-up of 2.4 years, compared with placebo, pioglitazone reduced the risk of conversion of impaired glucose tolerance to type 2 diabetes by 72% (95% confidence interval, 0.16–0.49; $P < 0.001$) [118]. Similarly, for the primary [120] and secondary [122] prevention of stroke, there was a similar magnitude of benefit with pioglitazone treatment. IRIS (Insulin Resistance Intervention after Stroke) was a primary prevention trial [120]. 3876 patients who had had a recent ischemic stroke or transient ischaemic attack, subjects received either pioglitazone (target dose, 45 mg daily) or placebo. Eligible patients did not have diabetes but were found to have insulin resistance on the basis of a score of more than 3.0 on the homeostasis model assessment of insulin resistance (HOMA-IR) index. The primary outcome was fatal or non-fatal stroke or myocardial infarction. By 4.8 years of follow-up, a primary outcome had occurred in 175 of 1939 patients (9.0%) in the pioglitazone group and in 228 of 1937 (11.8%) in the placebo group (hazard ratio in the pioglitazone group, 0.76; 95% confidence interval [CI], 0.62–0.93; $P = 0.007$). Diabetes developed in 73 patients (3.8%) and 149 patients (7.7%), respectively (hazard ratio, 0.48; 95% CI, 0.33–0.69; $P < 0.001$). Importantly, overall safety and tolerability was good with no change in the safety profile of pioglitazone identified. For example, in the high-risk PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events) [119], whilst there was a very slight increase in the well-recognised side effect of cardiac failure 6% versus 4% of those in the pioglitazone versus placebo groups; mortality rates from heart failure did not differ between groups. Thus, in summary in NAFLD, treatment with pioglitazone may directly benefit the liver and decrease risk of type 2 diabetes, myocardial infarction and stroke (Fig. 9.3).

Pioglitazone targets both adipose tissue metabolism and inflammation, acting through the transcription factor PPARγ. PPARγ has three splicing variant isoforms (1–3, and) that display differences in tissue localisation for each isoform: 1 (ubiquitous localisation), 2 (localised in adipose tissue) and 3 (localised in macrophages, colon and adipose tissue) [123]. PPARγ is predominantly expressed in adipocytes, immune cells including macrophages (and Kupffer cells) and hepatic stellate cells. PPARγ agonists activate PPARγ receptor function to decrease supply of fatty acids to the liver by promoting pre-adipocyte differentiation. Additionally in the liver PPARγ agonists activate Kupffer cells polarisation from a pro-inflammatory M1 to a pro-resolving M2 phenotype [124] and reverses hepatic stellate cell trans-differentiation to myofibroblasts [125].

Although it is uncertain whether pioglitazone has actions in the intestine, colonic epithelium expresses high levels of PPARγ3 receptors, and a high potency natural ligand for PPARγ3 receptors is butyrate [126]. Butyrate is produced locally in the large intestine from the gut microbiota-induced fermentation of carbohydrate. Thus, dysbiosis could adversely affect the integrity of intestinal permeability via a butyrate-PPARγ3-mediated effect, with a consequent increase in lipopolysaccharide concentrations in the portal circulation promoting the risk of NASH. Interestingly,

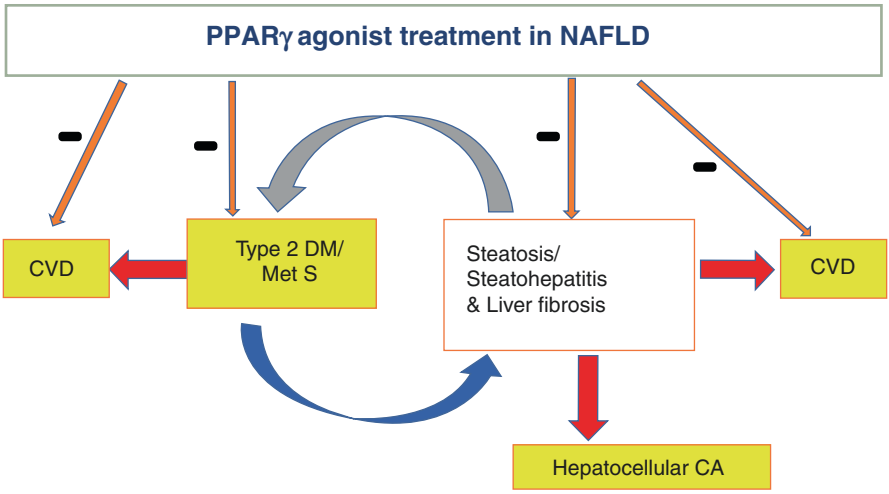

Fig. 9.3 PPARγ agonist treatment in NAFLD. NAFLD increases risk of type 2 diabetes, CVD and hepatocellular carcinoma. With the development of type 2 diabetes, there is a further increase in the risk of liver fibrosis. With development of more advanced forms of liver fibrosis (e.g. F3 and F4 fibrosis), there is a marked increase in the risk of hepatocellular carcinoma. In NASH, PPARγ agonist treatment has a beneficial effect to cause resolution of NASH in ~50% of patients (after 2 years of treatment). PPARγ agonist treatment may be also beneficial in reducing liver fibrosis (although further research is needed in this patient group). PPARγ agonist treatment decreases risk of type 2 diabetes (and lowers plasma glucose concentrations in patients who have established type 2 diabetes). PPARγ agonist treatment also decreases risk of myocardial infarction and stroke

in mice a high-fat diet modifies the PPAR-γ pathway leading to disruption of the microbial and physiological ecosystem in small intestine, and these effects were reversed by treatment with the PPARγ agonist rosiglitazone [127]. Since many patients with type 2 diabetes also have dysbiosis and NASH, PPARγ agonist drugs would seem an ideal form of treatment for this group of patients, particularly if it were possible to develop even better PPARγ agonists that retained the beneficial effects of the drugs without increasing the risk of known side effects associated with the class. The potential modes of action of PPARγ agonists in NAFLD are shown in Fig. 9.4.

There is considerable interest in determining whether it is possible to dissociate the benefits of pioglitazone from the side effects. In recent years the global usage of pioglitazone has plummeted, largely because of fears about side effects associated with this drug (such as increased risk of bone fracture, fluid retention and increases in body fat). It has been known for many years that post-translational modification (PTM) of the PPARγ in the form of altered phosphorylation status affecting functioning of the PPARγ receptor [128, 129] and PPARγ activity is also known to be regulated by other PTMs such as sumoylation and ubiquitinylation [130]. Kraakman et al. [131] have recently tested in mice whether another PTM, i.e. deacetylation of PPARγ, is able to dissociate the metabolic benefits of TZDs from

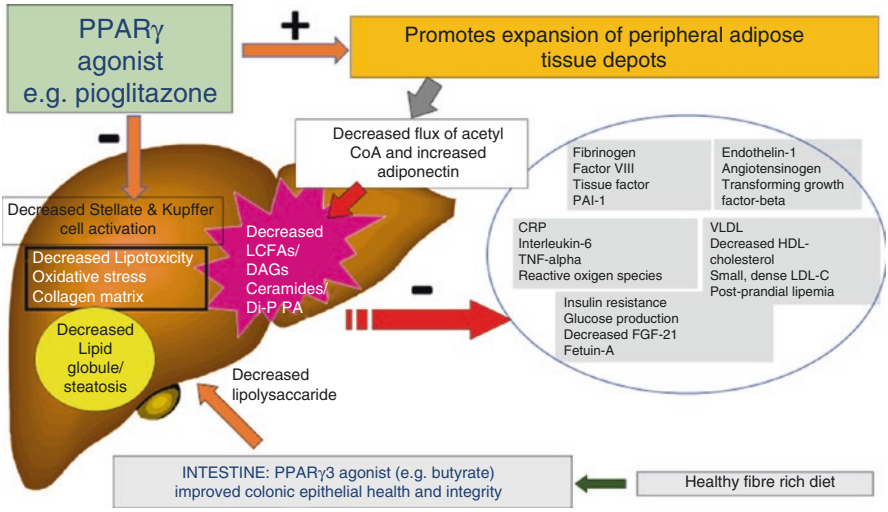

Fig. 9.4 Potential modes of action of PPARγ agonist effects to confer benefit in NAFLD. PPARγ agonist treatment acts via PPARγ2 receptors in pre-adipocytes to promote adipocyte differentiation, increase expandability of peripheral adipose tissue depots and increased adiponectin release from adipocytes. The increased expandability of peripheral adipose tissue depots decreases fatty acid flux to the liver with consequent decreased fluxes in acetyl Co-A for lipid synthesis. Increased adiponectin release potentially decreases inflammation and improves hepatic insulin sensitivity. PPARγ agonist treatment acts in liver via PPARγ3 receptors in macrophages (Kupffer cells) to decrease activation of macrophages and also acts in hepatic stellate cells to decrease activation of these matrix-producing cells. Although it is uncertain whether pioglitazone has actions in the intestine, colonic epithelium expresses high levels of PPARγ3 receptors and butyrate is a high-potency natural ligand for PPARγ3 receptors. Butyrate is produced locally in the large intestine from the gut microbiota-induced fermentation of carbohydrate. Thus improvements in dysbiosis, perhaps due to a Mediterranean diet (see Fig. 9.2), could improve intestinal permeability via a butyrate-PPARγ3-mediated effect. A potentially beneficial consequence of this effect would be reduced levels of lipopolysaccharide in the portal circulation, thus reducing the levels of this pro-inflammatory stimulus in the liver. *DAGs* di-acylglycerols, *di-P PA* di-palmitoyl phosphatidic acid, *LCFAs* long-chain fatty acids; *PAI-1* plasminogen activator inhibitor-1, *CRP* C-reactive protein, *TNF-alpha* tumour necrosis factor, *FGF-21* fibroblast growth factor, *VLDL* very-low-density lipoprotein, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density-lipoprotein cholesterol

their adverse effects. TZDs induce the deacetylation of PPARγ on K268 and K 293 to cause the browning of white adipocytes. By mutating these two lysine residues to arginine (2KR) there is constitutive deacetylation of PPARγ and increased energy expenditure protecting the mice from diet-induced obesity, glucose intolerance and hepatic steatosis. When 2KR mice were treated with the TZD rosiglitazone, they retained the beneficial response to the TZD without the evidence of the potentially harmful side effects of the drug such as bone demineralisation, fluid retention or fat deposition. Intriguingly, these data provide the first evidence that it is possible to dissociate benefits from harms with this class of drugs. The data also suggesting the following fascinating possibilities:

(a) it may be possible to design more specific PPARγ drugs than current TZDs or even to combine PPARγ drugs with specific acetylation inhibitors;
(b) variable acetylation status of the PPARγ receptor may be very important in normal physiological conditions to increase the risk of obesity and obesity-related conditions;
(c) and maybe lower doses of currently available TZDs (pioglitazone) would still confer a benefit in NASH without increasing the risk of harmful side effects.

9.4.2.2 Vitamin E

Although vitamin E is recommended for consideration of treatment of NASH in the Guidelines discussed above [102, 104, 132], there is less convincing evidence that vitamin E treatment confers any benefit beyond the liver. Although several observational epidemiologic studies suggested that vitamin E supplementation might decrease the risk of developing CVD, these data were not substantiated by the results from RCTs testing the effects of vitamin E on a variety of CVD end points.

Vitamin E is a powerful antioxidant that has the potential for reacting with lipid peroxyl radical and over the last two decades a number of studies have tested whether vitamin E is beneficial for CVD [133]. There have been several RCTs that have tested the effects of vitamin E on a range of CVD-related end points. Of these studies, notably the Physicians Health Study of 14,641 men over the age of 50 years were randomised to receive vitamin E (400 IU/day) alternate days and vitamin C (500 mg/day) every day for 8 years [134]. During a mean follow-up of 8 years, there were 1245 confirmed major cardiovascular events. Compared with placebo, vitamin E had no effect on the incidence of major cardiovascular events (both active and placebo vitamin E groups, 10.9 events per 1000 person-years; hazard ratio [HR], 1.01 [95% confidence interval, 0.90–1.13]; $P = 0.86$), as well as total myocardial infarction (HR, 0.90 [95% CI, 0.75–1.07]; $P = 0.22$), total stroke (HR, 1.07 [95% CI, 0.89–1.29]; $P = 0.45$) and cardiovascular mortality (HR, 1.07 [95% CI, 0.90–1.28]; $P = 0.43$). Importantly, vitamin E was associated with an increased risk of haemorrhagic stroke (HR, 1.74 [95% CI, 1.04–2.91]; $P = 0.04$). Further longer-term follow-up of this cohort with over 11 years of follow-up in 2012 confirmed there was no CVD benefit of vitamin E treatment [135]. Furthermore, a meta-analysis of the dose–response relationship between vitamin E supplementation and total mortality using data from RCTs was reported in 2005 [136]. Data from 135,967 participants in 19 clinical trials were analysed. Of these trials, nine trials tested vitamin E treatment alone and 10 tested vitamin E combined with other vitamins or minerals. The dosages of vitamin E ranged from 16.5 to 2000 IU/day (median, 400 IU/day). Nine of 11 trials testing high-dosage vitamin E (≥400 IU/day) showed increased risk for all-cause mortality in comparisons of vitamin E versus control. The pooled all-cause mortality risk difference in high-dosage vitamin E trials was 39 per 10,000 persons (95% CI, 3–74 per 10,000 persons; $P = 0.035$). A dose–response analysis showed a statistically significant relationship between vitamin E dosage and all-cause mortality, with increased risk of dosages greater than 150 IU/day.

Although the generalisability of these findings to patients with NAFLD is uncertain, considering that high-dosage (≥400 IU/day) vitamin E supplements may increase all-cause mortality and the dose of vitamin E that has been tested in NASH was 800 IU/day [115]; in our opinion vitamin E treatment should not be considered in NAFLD.

9.4.3 Statins

The role of statins in liver disease has recently been reviewed [137]. Although there had previously been concern that this class of agents may be harmful in liver disease, these agents are now known to be safe in patients with NAFLD. Analysis of the Dallas Heart Study data in 2006 showed that in 2264 Dallas Heart Study participants who were using no lipid-lowering agent ($n = 2124$), or who were being treated with a statin for lipid management ($n = 140$), statin use was not associated with a greater frequency of hepatic steatosis (38% vs. 34%) or elevated serum ALT (15% vs. 13%) by a pair-matched analysis [138]. A Cochrane Systematic Review in 2013 concluded that trials with larger sample sizes and low risk of bias are necessary before it could be concluded that statins were an effective treatment for patients with NASH. However, it was stated that because statins can improve the adverse outcomes of other conditions commonly associated with NASH (for example, hyperlipidaemia, diabetes mellitus, metabolic syndrome), the use of statins in patients with NASH may be justified [139]. A recent systematic review in 2017 evaluated the effects of statins in chronic liver disease [140] and found that statin use is probably associated with lower risk of hepatic decompensation and mortality, and might reduce portal hypertension, in patients with CLDs. Thirteen studies (3 randomised trials, 10 cohort studies) were identified in adults with chronic liver diseases, reporting the association between statin use and risk of development of cirrhosis, decompensated cirrhosis, improvements in portal hypertension, or mortality. Among 121,058 patients with CLDs (84.5% with hepatitis C), 46% were exposed to statins. In patients with cirrhosis, statin use was associated with 46% lower risk of hepatic decompensation (4 studies; RR, 0.54; 95% CI, 0.46–0.62), and 46% lower mortality (5 studies; RR, 0.54; 95% CI, 0.47–0.61). In patients with CLD without cirrhosis, statin use was associated with a nonsignificant (58% lower) risk of development of cirrhosis or fibrosis progression (5 studies; RR, 0.42; 95% CI, 0.16–1.11). In three randomised controlled trials, statin use was associated with 27% lower risk of variceal bleeding or progression of portal hypertension (hazard ratio, 0.73; 95% CI, 0.59–0.91). Thus one can conclude that prospective observational studies and randomised controlled trials are needed to confirm this observation.

Although, other agents have been tested in patients with NAFLD which have effects on CVD risk factors such as liraglutide, obeticholic acid and omega-3 fatty acids, none of these agents are currently recommended in international guidelines for patients with NASH. Because of the limitations of space and the remit of this chapter, we have therefore not discussed the use of these agents in NASH.

In summary, we consider that pioglitazone treatment should be considered for all patients with NASH, regardless of whether they have type 2 diabetes, providing the drug is not contraindicated. In our opinion it is important to undertake a baseline diagnostic liver biopsy and a repeat follow-up biopsy after ~2 years to evaluate response to pioglitazone therapy. For patients who show improvement of NASH, pioglitazone should be continued, and for patients who have worsening of NASH or no improvement of liver disease, the drug should be withdrawn. In contrast, for patients with NASH who also have co-existing type 2 diabetes, pioglitazone treatment should be used unless contraindicated, specifically as an effective glucose-lowering agent in this patient group. For such patients, pioglitazone treatment is advocated primarily as a treatment for type 2 diabetes with the possibility that it may also benefit liver disease in NAFLD, and decrease risk of myocardial infarction and stroke. For all patients with NAFLD, CVD risk should be assessed using available CVD risk calculators.

9.5 Conclusions

Since NAFLD is embedded in a more complex metabolic disease, it is difficult to dissect the independent role of a metabolically dysfunctional liver on the development of CVD. Therefore, the design of future clinical studies should take account of metabolic confounders such as adipose tissue dysfunction, metabolic inflammation, gut dysbiosis, dyslipidaemia and hyperglycaemia. Secondly, the different methods used for the diagnosis of NAFLD add uncertainty as to the relationship between liver disease severity and any association with CVD. Finally, some of the prospective epidemiological cohort studies had a long period of follow-up, without repeat measurements during follow-up which could result in confounding by unmeasured factors. Therefore, we suggest that better designed epidemiological studies are needed to clarify the independence of the role of the liver in NAFLD in the development of CVD.

We consider that two key questions still require further research. Firstly, which patients with NAFLD are at higher risk of CVD and secondly, do patients with CVD need to be screened for NAFLD?

With regard to the first question, as described in this chapter, the early anatomical and structural changes in the liver due to lipid accumulation in hepatocytes can cause a disruption of the hepatic sinusoids leading to non-cirrhotic portal hypertension. This process occurs before hepatic collagen deposition and therefore before the development of NASH [50]. Therefore, measurement of the hepatic venous pressure during liver ultrasound could be an inexpensive strategy that might help to identify a subset of patients with NAFLD at higher risk of developing myocardial dysfunction. However, further evaluation is needed in patients with NAFLD because measuring hepatic venous pressure could be recommended in the evaluation of CVD risk. Other tests include measurement of carotid ultrasound for measuring carotid intima-media thickness, flow-mediated dilation for measuring endothelial function, echocardiography for identifying any myocardial dysfunction and high-resolution

computed tomography for the estimation of coronary calcium score to detect early signs of coronary artery atherosclerosis, may have clinical utility in refining the estimation of cardiovascular risk in NAFLD, but discussion of their value in NAFLD is beyond the scope of this chapter.

With regard to the second question, we think that more research needs to be undertaken to identify the prevalence of NAFLD in patients with established CVD. Investigating abnormalities of simple liver function tests with measurement of liver fat with ultrasound, combined with the assessment of liver fibrosis with simple biomarker tests in those patients with diagnosed liver fat, would help the physician to identify NAFLD and assess liver disease severity in those patients with established CVD [141].

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Conflict of Interests All authors have no conflicts of interest to disclose.

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NAFLD, Hepatocellular Carcinoma, and Extrahepatic Cancers

10

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10.1 Introduction

Hepatocellular carcinoma (HCC) stands as the most overlooked complication of NAFLD and probably the most challenging in clinical practice. The large burden of the underlying liver disease, the chance of HCC arising in the absence of cirrhosis, and the incomplete knowledge of the mechanisms leading to carcinogenesis in NAFLD hampers the development of markers for targeting subjects at high risk and contributes to impede an effective care of patients with HCC.

10.2 Risk Factors for the Development of HCC in NAFLD

Beyond the well-known risk factors of underlying cirrhosis and male gender, several specific factors concur to increase the risk of HCC in NAFLD and translate into the unpredictable onset of cancer even in a non-cirrhotic liver.

10.2.1 Obesity and Type 2 Diabetes

Obesity and type 2 diabetes (T2DM) have a well-established, independent, and cumulative impact in the development of HCC, also in cirrhosis of viral and alcohol-related

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etiology [1, 2]. The likelihood of dying from liver cancer in men with a body mass index (BMI) of 35 kg/m^2 or above over 16 years of follow-up is increased by 4.5-fold compared to men with a normal BMI (18.5–24.9 kg/m^2) [1]. The association between BMI and liver cancer is more marked in men than women and the risk linearly increases starting from BMI above 22 kg/m^2 [3]. A meta-analysis of 11 cohort studies estimated that the risk of HCC is increased by 17% in overweight and by 89% in obese subjects, with an average 24% increase in risk for each 5 kg/m^2 increase in BMI [4].

The association of obesity and incident HCC has an ethnic specificity as it is observed in white Caucasian, Latino, and Asian men, but not in Afro-Americans [5]. Visceral fat accumulation is likely to play an important role, particularly in the non-obese population. The waist-to-hip ratio, a rough estimate of abdominal fat, can predict better than BMI the incidence of HCC [6].

Similarly to obesity, in diabetes the risk of HCC is on average increased by 20%, with a hazard ratio (HR) of 2.24 in males and 1.94 in females compared with non-diabetic subjects [7].

Among the single features of MetS, T2DM is associated with the highest risk for HCC (up to fourfold compared with nondiabetics), followed by obesity (up to twofold compared with non-obese). Combining multiple hallmarks of MetS, the risk of HCC increases in parallel with the number of features considered, reaching the highest risk (+475%) in patients who are overweight and diabetics [8]. Both obesity and T2DM per se exert a carcinogenic potential, but the underlying presence of NAFLD and NASH is usually underestimated, and HCC may be the presenting feature of a clinically insidious and asymptomatic liver disease.

10.2.2 Genetic Background

Multiple risk factors related to host phenotype significantly interact with the genetic background to increase the risk of malignancy (see also Chap. 8). The PNPLA3 rs738409 [G] risk allele, found in 40% of the European population, is repeatedly reported to increase about 12-fold the risk of developing HCC [9]; further, among HCC patients, GG homozygosity is also associated with younger age, shorter history of cirrhosis or less advanced liver disease, and more diffuse HCC at diagnosis, hence reduced survival. Other uncommon genetic variants seem to influence HCC development in a fatty liver. The most important is the human telomerase reverse transcriptase (TERT) gene, which is upregulated in human cancer and is a hallmark of HCC in patients carrying loss-of-function TERT mutations [10].

10.2.3 Other Risk Factors

The combination of metabolic and genetic risk factors above described could be a fertile soil for the malignant degeneration of benign liver lesions, such as hepatocellular adenomas (HCA), also in the absence of cirrhosis. There is an association

between the rising prevalence of obesity and MetS and the recent increase in the HCA prevalence, more likely inflammatory (I-HCA) [11, 12]. Obesity and MetS has been often associated with multiple and bilobar adenomas, leading to a higher rate of incomplete resection, and with progression of HCA; conversely, stability or regression of tumor burden is described in up to one-third of patients complying with lifestyle changes (weight loss >5%) [11]. In a French study which analyzed 31 HCC patients who had MetS as the only risk factor, one-third of these cases developed in a preexisting hepatocellular adenoma [13], while a literature review of 1600 adenomas showed that nearly 4% of them presented HCC features at the time of resection [14].

10.3 Epidemiology of HCC in NAFLD

NAFLD is the source of HCC most rapidly increasing, in parallel with the spread of obesity and diabetes across the general population [15, 16]. It is necessary to recall that NAFLD may remain unrecognized in cases of HCC arising in cryptogenic cirrhosis, a condition for which no underlying etiology has been clinically identified. It is estimated that 20–40% of all HCC cases in industrialized countries occur in patients with cryptogenic cirrhosis. Most of the cases have been identified as "burnout NASH," bearing historical or metabolic vestiges of MetS but no longer having classic biopsy features (31, 32 di ARM), which often disappear in cirrhosis.

In the United States, NAFLD represented the third most common cause of HCC, after hepatitis C and alcohol-related disease, being diagnosed in 14.1% of patients with HCC [17]. In North-East England, HCC associated with NAFLD had a more-than-tenfold increase in between 2000 and 2010, accounting for 35% of all the cases of HCC [18]. Hence, it is not surprising that NAFLD is the most rapidly increasing indication for liver transplantation (LT) due to HCC in USA, where from 2002 to 2012 the number of NAFLD-related HCC increased by 365% and become the second leading cause of LT after HCV-related cases [19]. The growing importance of HCC arising in NAFLD will become obvious after the decline of HCV infection thanks to direct-acting antivirals therapy although it will take decades to occur.

The risk of HCC occurrence in NAFLD is lower than in chronic hepatitis C. Overall, the 1-year cumulative incidence of HCC in patients with NAFLD has been estimated at around 2.5% compared with 4% in patients with hepatitis C, while the 5-year incidence rises to 11% and 30%, respectively [20]. However, the lower prevalence and incidence of HCC in NAFLD must be outweighed by the much larger spread in industrialized countries and the steady rise of its risk factors also in developing countries. Importantly, the alarming growth of NAFLD in the pediatric population can bear an increased risk of liver-related complications in adulthood. A longitudinal study [21] including Danish schoolchildren aged 7–13 years showed that each unit increase in BMI *z*-score will rise by 20–30% the risk of liver cancer 30 years later. Similarly, a study in the USA [22] reported that each unit increase in BMI in the mid-twenties can hasten by 4 years the occurrence of liver cancer,

confirming that obesity in early adulthood is associated with increased risk of developing HCC at a younger age in the absence of other relevant risk factors. These data highlight once again the importance of a global policy for the prevention of obesity and its related complication since childhood.

10.4 HCC in Non-cirrhotic NAFLD

HCC can also develop in non-cirrhotic NASH, with at least 116 such cases reported so far since 2004. The initial observation had been made in a single-center pathological study on 128 patients undergoing liver resection for HCC between 1995 and 2007 [23]; HCC arising in livers without significant fibrosis occurred more frequently in patients with metabolic syndrome (MetS) and NAFLD (65.5%) than in patients with known liver disease of other origin (25%). This peculiar feature has been afterwards confirmed by epidemiological studies. In a U.S. Veterans Administration cohort [24], the risk of HCC in the absence of cirrhosis was fivefold higher in patients with NAFLD, compared to those with chronic hepatitis C. In a tertiary center for HCC referral in Northern England [18], those with NAFLD as underlying liver disease had a lower prevalence of cirrhosis (77.2%) compared with other etiologies. As these patients were not in surveillance programs, the majority (62.3%) presented symptomatically, with larger tumors, and their median survival was just 7.2 months.

10.5 Molecular Mechanisms of Carcinogenesis

Some HCC developing in NAFLD patients could belong to a particular subtype of hepatic tumors with distinct histological features, called "steatohepatitic hepatocellular carcinoma," characterized by histological hallmarks resembling steatohepatitis, such as steatosis, hepatocyte ballooning, Mallory bodies, and peri-hepatocellular fibrosis [25]. Mechanisms linking the progression of steatosis to HCC, with or without cirrhosis, are probably more related to the pathogenesis of the underlying disease rather than to fibrosis, with an important role attributed to environmental factors leading to obesity and diabetes (Fig. 10.1). The common soil of insulin resistance (IR) and hepatic steatosis favors liver carcinogenesis by promoting adipose tissue-derived inflammation, hormonal changes, oxidative stress and lipotoxicity, and stimulation of the IGF-1 axis by hyperinsulinemia. Other mechanisms involving diet, gut microbiome, and genetic factors are increasingly important. Western high-fat diet can induce the expression of cytokines like IL-6 and TNFα and increase NF-κB activation [26]. Fructose may play an important role by increasing lipoperoxidation [27], downregulating the expression of sirtuin-1, involved in the regulation of cellular survival, or altering the intestinal microbioma composition [28]. Gut microbioma contributes to hepatic inflammation by increasing intestinal permeability, promoting translocation of bacterial components such as lipopolysaccharides and favoring the activation of the toll-like receptors.

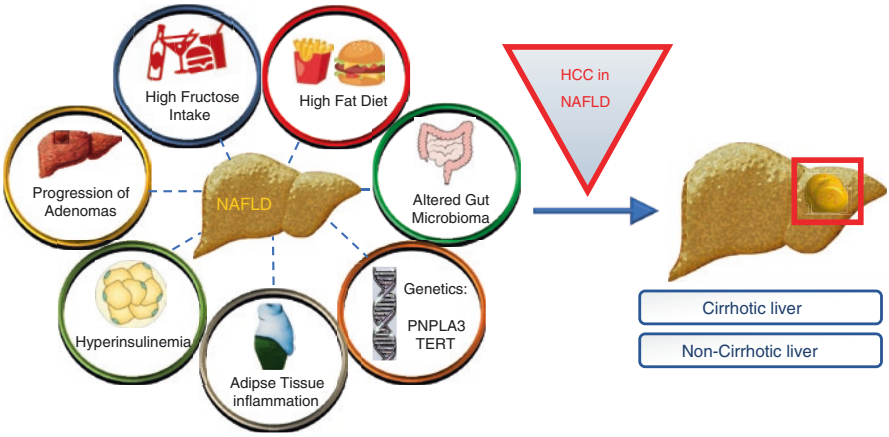

Fig. 10.1 Mechanisms promoting the onset of HCC in NAFLD

10.6 Surveillance of HCC

Poor surveillance is a constant problem for NAFLD patients, and liver-related complications can be the presenting features. Approximately 60% of patients with HCC related to NAFLD missed regular surveillance resulting in more advanced HCC burden at diagnosis compared to patients with hepatitis C [29, 30]. Systemic surveillance for HCC is currently impracticable in patients without cirrhosis. In a retrospective analysis, 86% of patients with HCC in non-cirrhotic liver had a larger nodule size and/or a greater rate of recurrence compared with 14% of patients with HCC in cirrhosis [13], leading to a reduced chance of curative treatment [31].

Programming an optimal screening strategy for the early detection of HCC in NAFLD is not trivial because of the burden of potential candidates for systemic surveillance (including non-cirrhotic patients) and the absence of reliable non-invasive tools and molecular signatures able to stratify the risk in the NAFLD population. In consequence, specific recommendations are lacking in current guidelines. The practice of oncologic follow-up on an individual basis is supported by three out of five guidelines on the management of NAFLD [32–34]. The guidelines of the Asia-Pacific region suggest the extension of screening to those "cancers whose incidence is increased by MetS," but without a generalized and standardized program. The most recent EASL-EASD-EASO clinical practice guidelines still indicate that the large number of NAFLD cases at risk of HCC makes systematic surveillance largely impracticable [32]. In the current guidelines for management of HCC, surveillance is recommended in NAFLD patients with cirrhosis only, according to standard practice (twice-yearly ultrasound examination of the liver) [33]. Surveillance is deemed as cost-effective if the expected risk for incident HCC exceeds a threshold of 1.5% per year, but epidemiological studies in non-cirrhotic NASH are still inadequate to answer this question [33, 35]. Of note, surveillance by abdominal ultrasound has a suboptimal performance in NAFLD patients, with a high rate of under-recognition

of small nodules [36, 37]. Recently a scoring system based on age, sex, medical history of diabetes and viral hepatitis, aminotransferases, and α-fetoprotein was able to identify almost all HCC cases detected by ultrasound in Taiwan high-risk patients [38].

10.7 Prevention of HCC

Despite many uncertainties, available knowledge suggests that HCC in NAFLD develops slowly during a lifetime; however, the earlier the exposure to risk factors, the earlier the onset of malignancy, particularly on a genetically predisposed background. Hence, prevention of obesity starting from childhood should be a priority in the agenda of educational programs and of health-care providers. Lifestyle modification can be able itself to change the natural history of the disease. In a prospective cohort study in Taiwan analyzing risk prediction models for HCC (n = 428,584, HCC = 1668) during an average follow-up of 8.5 years, physical activity reduced the risk of developing HCC proportionally to the intensity of exercise and regardless to the etiology of liver disease [38]. Secondly, effective therapies to cure NASH can reduce the burden of patients at high risk for developing HCC. In the last years, the landscape of potentially curative treatments is rapidly growing, as reviewed in Chapter 17.

Among old drugs often prescribed in NAFLD patients, metformin seems to enhance antitumor mechanisms by mTOR inhibition [39]. In a meta-analysis, the use of metformin in 105,495 patients with T2DM was associated with a reduction of 50% in HCC risk, while the risk was increased when sulfonylurea or insulin was used [40]. Further, metformin seems to improve the outcome of HCC treatment: in a prospective Taiwanese study in diabetic patients with early stage HCC undergoing radiofrequency ablation, a lower mortality rate was observed in patients under metformin [41]. Statins may also decrease the risk of cancers through antiproliferative, proapoptotic, antiangiogenic, and immunomodulatory effects. A systematic meta-analysis from 26 randomized, controlled trials, including almost 1.5 million patients and 4298 cases of HCC, showed that the use of statins was associated with a 37% reduction in HCC incidence after adjusting for potential confounders [42]. All these data suggest that the use of these medications should be encouraged in patients with NAFLD beyond their metabolic and cardiovascular benefits.

10.8 Treatment of HCC

The therapeutic options for NAFLD-related HCC are the same as those for any patient with liver disease (LT, resection, radiofrequency ablation, chemoembolization, sorafenib) [35], but late diagnosis, older age, and concurrent metabolic or vascular disease restrict the options for potentially curative treatments. Furthermore, loco-regional treatments can be limited by technical difficulties in ultrasound detection or by peripheral atherosclerosis, while increased risks for infection, metabolic

decompensation, and cardiovascular complications can hamper surgical options [43]. The drawbacks of older age, higher rate of cardiovascular and metabolic comorbidities, and higher rate of unresectable HCC can be partially outweighed by a better preserved liver function or a lower prevalence of cirrhosis in these patients. In a retrospective cohort in USA assessing the outcome of curative treatments for HCC [10], NAFLD patients had a better hepatic synthetic function than patients with hepatitis C or alcohol-related liver disease, and were more likely to undergo liver resection (41% in the NAFLD group compared with 13% in the hepatitis C and alcohol-related liver disease group, $p = 0.002$).

In accordance with these data, recent findings suggest that NAFLD patients do not have a different morbidity and mortality compared to other etiologies after surgical treatment for HCC. In a study which evaluated the outcome of HCC treatment in 303 patients from 2000 to 2010, after a median follow-up of 50 months, no difference was found in recurrence-free survival and overall survival between NAFLD and HCV or alcohol-related HCC, independent of other pathologic factors and type of curative treatment [10]. Regarding liver transplantation, from 2002 to 2012, the indication for LT in patients with HCC and NAFLD has increased by nearly fourfold compared to a twofold increase in those with HCV-related HCC [19]. However, patients with NAFLD are less likely to receive a liver graft than patients with HCV or alcoholic liver disease [44]. Very high BMI, especially morbid obesity, represents a contraindication to LT, and some centers begin to consider obesity treatment like bariatric surgery as preparation for LT. Even though it might be difficult or impossible in patients with end-stage liver disease, preliminary results suggest that combined LT along with sleeve gastrectomy might be considered in selected cases [45]. After LT, the 5-year survival in NAFLD does not differ from non-NAFLD because the greater risk of death from cardiovascular complications and sepsis is outweighed by a lower risk of graft failure [46].

10.9 NAFLD and Extrahepatic Cancers

The second most common cause of death among NAFLD patients is attributed to malignancies at either gastrointestinal (liver, colon, esophagus, stomach, and pancreas) or extraintestinal sites (kidney in men and breast in women) [47–50]. Although the evidence is still preliminary, the colon is the main extrahepatic site where a link between NAFLD and cancer seems to be consistent. Most studies, both community-based and hospital-based, have been conducted in East Asia. Almost all of these studies showed a higher prevalence of colorectal lesions in patients with NAFLD compared to patients without NAFLD. In a large retrospective cohort study of 5517 Korean women, Lee et al. observed a twofold increase in the occurrence of adenomatous polyps and a threefold increase in the risk of colorectal cancer (CRC) in patients with US-diagnosed NAFLD compared to controls [51]. The risk of CRC is further increased in NASH. In a Chinese study, NASH patients harbored a fivefold increased risk of both adenomas (OR 4.89) and advanced neoplasms (OR 5.34) even after adjusting for demographic and metabolic factors [52]. Importantly,

a significant proportion of lesions developed in the proximal colon and at a much younger age. In Caucasians, much less data are available. In a large European study ($n = 1382$), Stadlmayr et al. observed that male patients with US-diagnosed NAFLD had a higher prevalence of colorectal adenomas and early CRC compared to those without NAFLD, and the increased risk (OR 1.47) was independent of other known factors [53].

The role of fatty liver in the increased risk of CRC is purely speculative. A generic increased risk of cancer in NAFLD is common to all the components of MetS and is due to increased insulin and insulin growth factor (IGF) levels [54], which exert their normal activity as growth factors and stimulate cell proliferation, apoptosis, and production of vascular endothelial growth factor [55]. Conversely, decreased adiponectin levels restrain its proapoptotic activity and anticarcinogenic action [56, 57]. The increased pro-inflammatory state characteristic of NASH may further influence apoptosis and tumor cell proliferation [58, 59]. However, the increased risk of cancers in the bowel rather than in other sites does not appear casual in NAFLD as the liver stays at the cross-road of the complex interaction between IR and gut microbiota. A dysbiotic microbiota can promote tumorigenesis through chronic inflammation, increased interleukin-6 (IL-6) signaling, and decreased inflammasome-derived interleukin-18 (IL-18), which confers protection against tumors. Several bacterial metabolites, including hydrogen sulfide, secondary bile acids, polyamines, and reactive oxygen species (ROS), have the potential to cause deoxyribonucleic acid (DNA) damage or local inflammation via IL-6 and TNFα production, promoting carcinogenesis. Although it is still early to provide evidence-based recommendations, NAFLD patients should be a target group for CRC screening to reduce its incidence and mortality [52].

10.10 Future Directions

In consideration of the spread of obesity and NAFLD in the general population, the growing incidence of HCC can become a serious challenge for public health, with high costs for surveillance and treatment, including LT. Importantly, a considerable number of NAFLD-associated HCC cases develop in non-cirrhotic livers, particularly in patients with multiple metabolic risk factors. Delay in diagnosis and the presence of relevant comorbidities often limit the possibility of therapeutic intervention. Although weight loss can generally ameliorate obesity-induced complications, the capability to prevent the development of HCC or halt its progression is unknown. Many other questions remain to be answered, including the best strategy for targeting high-risk subjects in the general population. A better understanding of the molecular events leading from obesity to NASH and HCC will allow the discovery of new targets for therapeutic and preventive intervention. In the meanwhile, the best and probably sole effective intervention to address this growing problem is to hinder the spread of obesity and NAFLD through public awareness and education programs.

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NAFLD in Children: Implication for the Future

11

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11.1 Introduction

The epidemic of obesity has resulted in a parallel incremental burden of nonalcoholic fatty liver disease (NAFLD) worldwide. Also, in children and adolescents, NAFLD represents the most common cause of chronic liver disease in industrialized countries [1]. NAFLD in children is now considered a metabolic condition, which is strongly associated with the other metabolic features, such as hypertension and insulin resistance, increasing the risk of developing type 2 diabetes mellitus, metabolic syndrome, and cardiovascular disease at a young age [2, 3].

The exact prevalence of pediatric NAFLD is actually unknown, but available data describe a prevalence ranging from 3 to 12% in the general pediatric population, with peaks of 70% in obese children. In Western countries, the prevalence of NAFLD is estimated to be around 20–46%, while in Asian children the prevalence is lower, about 5–18%. Moreover, in Asia and Pacific Islands, significant differences are reported between urban and rural populations with a prevalence of 16–32% in urban areas versus a prevalence of 9% in rural populations. Obesity-related NAFLD was reported in 77% of obese/overweight Chinese children. In Australia, the prevalence of pediatric NAFLD was estimated to be approximately 10% in the total population and 27.6% among overweight and obese children [4]. These ethnic differences may be related to genetic, environmental, or sociocultural factors as well as differences in body composition, insulin sensitivity, and adipocytokine profile.

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As it is note, NAFLD is characterized by accumulation of fat in the hepatocytes (>5%) in the absence of other causes of liver steatosis, such as Wilson's disease, deficiency of alfa-1-antitripsin, celiac disease, autoimmune hepatitis, HCV infection, metabolic disorders, and alcohol or drug consumption. The simple hepatic steatosis is usually a benign condition, but in some cases, it may progress to more advanced forms of liver injury, characterized by the presence of inflammation and various degrees of fibrosis [nonalcoholic steatohepatitis (NASH)] up to cirrhosis, predisposing to liver failure and/or hepatocellular carcinoma (HCC) [2].

The natural history of pediatric NAFLD remains to be fully understood, considering the paucity of data available at medium/long-term and the complex interplay between liver involvement and other obesity-related metabolic impairments [5]. Several studies reported that children with NAFLD observed in a tertiary center have a significantly shorter survival compared to the general population of the same age and sex, and that, in some cases, NAFLD in children may progress to cirrhosis and end-stage liver disease [5].

In this chapter, the clinical, diagnostic, and therapeutic strategies of NAFLD currently known will be discussed, with particular interest to novel future scenarios in diagnosis and therapeutic approach to pediatric NAFLD.

11.2 Pathogenesis

In the last two decades, several advances have been made in the knowledge of pathogenesis of NAFLD. The two-hit hypothesis has been exceeded by the multi-hit theory, in which several agents have been identified as contemporary actors in the onset and progression of liver damage. Also in this theory, the starting point is represented by hepatic steatosis, which can be the result of several possible factors, such as dietary habits, environmental and genetic factors, insulin resistance, obesity with adipocyte proliferation, and changes in the composition of intestinal microbiota [6]. Subsequent noxae, such as pro-inflammatory cytokines, oxidative stress, circulating endotoxins, and activation of hepatic stellate cells, contribute to the progression of liver damage.

Adipose tissue is a metabolically active endocrine organ that causes the release of pro-inflammatory cytokines, such as TNF-α and IL-6, whereas beneficial adipokines are suppressed. This situation leads to the development of peripheral insulin resistance and hyperinsulinemia and increased fatty acid delivery to the hepatocyte. The disruption of normal insulin signaling in the hepatocyte and increased abundance of fatty acids leads to disordered lipid metabolism, characterized by the over-activation of de novo lipogenesis (DNL) transcriptional factors, causing more fatty acid and glucose products to be shunted into these lypogenetic pathways [7].

The role of intestinal microbiota has been recently considered within this metabolic dysregulation. A bad "obesogenic" diet (rich in fats and lipids) and increase of intestinal bacteria products (i.e., endotoxins, proteins, metabolites,

lipopolysaccharides (LPS)) with the subsequent activation of the toll-like receptor (TLR) pathway, may act as promoter of inflammation and progression of hepatic steatosis to NASH and fibrosis. This process seems also be aggravated by the increased intestinal permeability that has been demonstrated in subjects with liver disease, where the gut seems to go through a tight junction disruption process that could be reversed by changes in the microbiota composition.

These discoveries in the pathogenetic mechanisms are particularly relevant because of the possible therapeutic implications of prebiotics/probiotics and dietetic supplements in the treatment of NAFLD/NASH [8, 9].

11.3 Diagnosis

NAFLD is generally asymptomatic and the diagnosis is frequently made following the incidental discovery of hypertransaminasemia and/or hepatic steatosis. Hypertransaminasemia with a mild elevation of liver enzymes is a common finding in pediatric NAFLD, even if it is clearly demonstrated that aminotransferases serum levels may be in the normal range in several cases, independently from severity of liver damage [10]. However, serum ALT and AST concentration remains a cheap test for initial evaluation of NAFLD, even if its sensitivity is unsatisfactory. To make diagnosis of NAFLD, it is important to exclude other possible causes of hepatic steatosis and therefore an adequate screening panel should be done in all patients. Moreover, considering the strict association between NAFLD and metabolic syndrome, evaluation of metabolic parameters such as lipid profile, glyco-insulinemic status, uric acid concentrations, and blood pressure measurements are strongly recommended in patients with NAFLD [11].

A recent position paper by the ESPGHAN Hepatology Committee has clarified the diagnostic approach to NAFLD in childhood. NAFLD is more frequent in children aged more than 10 years and is usually present with overweight/obesity. As previously stated, the diagnosis of NAFLD needs the recognition of fatty liver and the exclusion of other causes of liver steatosis [2].

Liver biopsy remains the current gold standard for the diagnosis of NAFLD, and it is the only way to distinguish between NASH and simple steatosis, and to determine the presence and characteristics of fibrosis and the severity of liver damage. However, since liver biopsy is an invasive procedure, its use should be limited to selected patients, as stated by ESPGHAN Hepatology Committee [2].

In the last two decades, several biomarkers have been tested as diagnostic tools for NAFLD/NASH, but till now none of the markers evaluated is completely satisfactory in the diagnostic work-up. The major limitation of all these tests is the incapacity to estimate the severity of disease and the presence of fibrosis []. Higher levels of C-reactive protein and pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6, have been associated with progression of damage from NAFLD to NASH, but these markers lack specificity for NAFLD.

Recently cytokeratin-18, a marker of hepatocyte apoptosis, fragment levels, and the cathepsin-D (a lysosomal protease) were identified as reliable markers of NASH. In fact, it has been demonstrated that the cathepsin-D have a high diagnostic value to distinguish pediatric patients with hepatic inflammation from children with steatosis, while the cytokeratin-18 correlates significantly with hepatic fibrosis and with NAFLD severity [12, 13].

Another new marker of liver disease is the cathepsin D (CATD). The CATD is a proteolytic enzyme contained in the lysosomes of eukaryotic cells. The CATD are involved in autolysis processes, causing cleavage of the protein cellular constituents into peptides and amino acids. They also have the function of eliminating the extracellular proteins, such as the bacterial residues and the products of the antigen–antibody reaction [14]. A pediatric study of 2015 has shown that the plasma levels of CATD levels were significantly lower in patients with liver inflammation than those with only steatosis [15]. Moreover, the levels of CATD have been progressively reduced and negatively correlated with severity of liver inflammation, steatosis, hepatocellular ballooning, and the score of hepatic steatosis nonalcoholic activities. In addition, the levels of CATD are better correlated with the progression of nonalcoholic hepatic steatosis pediatric compared to ALT and CK-18 [14]. CATD showed a high diagnostic accuracy, with AUROC = 0.94, for the differentiation between hepatic steatosis and inflammation, and has reached nearly the highest precision (AUROC = 0.99) after addition of CK-18 [15]. The observation that plasma CATD correlated with the development of NASH and regression is promising for NASH diagnosis, but especially for the liver inflammation, which proceeds the onset of fibrosis.

Recently, genome-wide association studies (GWAS) have identified single-nucleotide polymorphisms (SNPs) of genes implicated in NAFLD/NASH pathogenesis capable to influence liver damage and fibrosis progression in adult and pediatric patients. Firstly, the PNPLA3, also known as adiponutrin, is a member of the patatin-like phospholipase family. The rs738409 C>G single-nucleotide polymorphism, encoding the Ile148Met variant protein of PNPLA3, is described as genetic determinant of hepatic steatosis. Several studies have established a strong link between PNPLA3 and the development of NAFLD [16]. PNPLA3 is associated with an increased risk of advanced fibrosis among patients with a variety of liver diseases and is an independent risk factor for hepatocellular carcinoma (HCC) among patients with NASH [17]. Besides PNPLA3, combined GWAS datasets have identified other SNPs associated with liver fat content and other aspects of the NAFLD phenotype, involving genes implicated in insulin resistance, oxidative stress, and fibrogenesis. For diagnosis and risk stratification of NAFLD, genetic screening tests are now available; these tests are easy to perform and have a low cost and can assess the risk of the subject of developing severe forms of NAFLD. Currently, a simple oral swab that searches for mutations in a combination of four genes (KLF6, PNPLA3, SOD2, and LPIN1), each of which is related to NAFLD, is able to estimate the risk of development of severe form of hepatopathy [18, 19].

The most used technique in the diagnostic work-up of NAFLD is abdominal ultrasound. It is widely used because it is simple, economic, and widely available. Unfortunately, US examination diagnose liver steatosis, only if fat is present in 30% of hepatocytes, and it do not detect minor degree of steatosic infiltration (<30%). Overall, the sensitivity of ultrasound in NAFLD ranges from 60 to 94%, with specificity from 84 to 100% [20]. The main limitation of liver ultrasound is represented by the inability in distinguishing between NAFLD and NASH due to its incapacity to detect liver fibrosis. This limitation is partially exceeded by transient elastography, an ultrasonographic technique based on the evaluation of tissue elasticity through ultrasound by measuring the propagation of specific elastic waves (shear waves, S-waves) emitted by the probe. However, abdominal obesity may reduce its utility in patients with NASH, and the detection accuracy of this method increases with worsening grades of fibrosis. For these reasons, it is not standardized in pediatric patients, and large studies are required to define normal values and the accuracy of transient elastography in children [21, 22].

Acoustic force impulse imaging radiation (ARFI) is an integrated US elastography method in US conventional machines in which a region of interest in the liver is mechanically excited with an acoustic pulse that induce localized tissue displacement, which translates in wave propagation. The wave propagation velocity correlates with liver stiffness and fibrosis. In a meta-analysis in patients with NAFLD (n = 77), the ARFI imaging had AUROC of 0.86 for the diagnosis of fibrosis [22].

Shear-wave elastography (SWE) are new methods that evaluate elasticity of the tissues using conventional USA. They allow the operator to select a specific area of the liver parenchyma, avoiding inclusion of focal lesions or large blood vessels and overcome some limitations of transient elastography. Acoustic imaging radiation impulse force showed good results in the differentiation stages of fibrosis in adults, especially in adult patients with chronic viral hepatitis. A recent study tested the SWE in 68 children with biopsy-proven NASH (37 males, age 8–17 years), demonstrating that SWE showed a high correlation with hepatic fibrosis (rho = 0.84, $p < 0.001$). The diagnostic accuracy in the determination of the presence of any grade of fibrosis showed an AUC of 0.92 (cutoff = 5.1 kPa), whereas the presence of significant fibrosis could be established with an AUC of 0.97 (cutoff = 6.7 kPa). These results are in line with a previous study of those that evaluated the accuracy of transient elastography in another pediatric NASH population [23].

Computed tomography (CT) and magnetic resonance imaging (MRI) are more sensitive and specific than ultrasound in determining liver steatosis, but these techniques have some limitations in pediatric setting. Firstly, both are expensive and not always widely available in pediatric setting. Moreover, CT implies an unjustified radiation exposure and MRI in several cases needs of sedation of pediatric patients. In addition, neither MRI nor CT are able to stage the disease and cannot distinguish between NAFLD and NASH, given that they could not detect hepatic fibrosis [2].

11.4 Treatment

Considering the actual knowledge on natural history of NAFLD/NASH in adults and children and health burden of metabolic comorbidities generally associated to NAFLD, the effective treatment of these conditions is now become a public health problem, mainly in children. The final goals of ideal treatments are to reverse hepatic histological damage, reducing long-term hepatic and extrahepatic complications and improving patient's quality of life and life expectation. Today, lifestyle intervention, based on hypocaloric diet and regular physical exercise, continues to be the basilar therapeutic approach, even if its results are often unsatisfactory, especially for the difficult compliance. Unfortunately, none of the drugs until now tested in children have proven to be completely adequate in the treatment of liver disease and therefore novel possible targets or pharmacological associations direct against the novel pathogenetic mechanisms are now being evaluated.

11.4.1 Actual Therapeutic Approaches

Gradual weight loss, due to behavioral modifications based on balanced diet and regular physical exercise, is still considered the first therapeutical approach to fatty liver in children. The available data demonstrated in several adults and pediatrics reports the effectiveness of this approach in terms of hepatic and extrahepatic insulin sensitivity, inflammation, and oxidative stress.

The main problem of this therapeutic strategy in pediatric NAFLD is represented by the difficulty in maintaining compliance of children and their families with the proposed programs, with disappointing results. Some reports described an inacceptable success rates as low as 10% after 2 years of intervention [24]. No clear indications are established for diet in pediatric patients with NAFLD, but a balanced diet containing low fat meats and fish, vegetables, legumes, and fruits with reduction in sugar-sweetened beverages, saturated fat, starches, and salt is generally recommended [25]. Moreover, in the last decade several data have demonstrated the toxic effect of high fructose intake on metabolic status and liver damage; therefore, a significant reduction of daily fructose intake is now strongly recommended [26].

As for pharmaceutical *armamentarium*, till now none of the available drugs has been revealed totally satisfactory *per sé* in the treatment of NAFLD. Starting from the new information gradually emerging about pathogenetic mechanisms of NAFLD, several molecules have been proposed, without however conclusive recommendation. The main limitations of many available studies are the frequent small sample size and the heterogeneity of diagnostic tools used to detect NASH and the different outcome measures. Only few randomized controlled clinical trials (RCTs) have been made or are ongoing in pediatric population.

- *Insulin-sensitizing agents.* Based on the strict interplay between insulin resistance and metabolic and liver damage typical of NAFLD/NASH, insulin sensitizer have been the first drug evaluated in children. Metformin activates the 5′adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway, which increases lipid and glucose catabolism. Recently, the large clinical TONIC trial, which enrolled 173 patients, reported that metformin (500 mg twice daily) caused only minor reductions in serum transaminase levels and had no significant effect on liver histology [27].
- *Antioxidants*. In the pathogenetic mechanism of NAFLD, the reactive oxygen species, generated by fatty acids oxidation, cause direct cellular damage and activate pro-inflammatory cytokines. The main antioxidant tested in children has been vitamin E (alpha-tocopherol), a fat-soluble vitamin. Initial small studies showing some efficacy of vitamin E in reducing transaminases levels were not confirmed in the TONIC trial, in which vitamin E was no better than placebo in attaining the primary end point, that is, a sustained decrease in ALT levels. Likewise, there was only a limited effect on hepatocellular ballooning and NAFLD activity score [27].
- *Dietetic supplementations*. Polyunsaturated fatty acids (PUFA) are long-chain fatty acids, distinguished in two groups: omega-3 PUFA, which are synthesized from α-linolenic acid, and omega-6 PUFA, which derive from linoleic acid. These acids are also defined "essential" fatty acids because they cannot be synthesized de novo by cells of human body and therefore should be taken in adequate amount with the diet. The omega-3 PUFA are important regulators of hepatic gene transcription, with anti-inflammatory, insulin sensitizing, and antisteatotic effects [28]. On the contrary, the metabolites derived from omega-6 PUFA pathways induce pro-inflammatory and pro-thrombogenic activation.

As it is well-known, the actual Western diets are characterized by a higher consumption of omega-6 fatty acid respect to omega-3 fatty acid, with a consequent imbalance between omega-6/omega-3. Several data have demonstrated, in animal and human studies, beneficial effects of omega-3 supplementation in hepatic and metabolic features of NAFLD. Particularly, Nobili and coworkers have described in well-conducted double-blind randomized controlled trial that oral administration of docosahexaenoic acid (DHA) in pediatric NAFLD causes at 6 and 24 months an amelioration of transaminases and triglycerides concentrations, insulin sensitivity index, and hepatic steatosis [21].

Based on the new advances in NAFLD pathogenesis, mainly following the development of "gut–liver axis theory," probiotics have been proposed as a possible strategy for treatment of liver disease, such as NAFLD. As previously stated, poor diet and slowed intestinal transit, frequent in obese patients, may induce small intestinal bacterial overgrowth (SIBO) and thereby increase the release of endotoxins [mainly gut-derived lipopolysaccharides (LPS)] and tumor necrosis factor (TNF)-α. These inflammatory mediators easily cross the intestinal barrier, which is

more permeable in patients with NAFLD, and promote the progression of NAFLD to NASH with a profibrogenic phenotype [29]. VSL#3, a mixture of eight probiotic strains (*Streptococcus salivarius* subsp. thermophilus, Bifidobacterium [*B. breve*, *B. infantis*, *B. longum*], *Lactobacillus acidophilus*, *L. plantarum*, *L. casei*, and *L. delbrueckii* subsp. *bulgaricus*) has been tested in animal models and human studies that include children. These studies show a beneficial effect on the intestinal barrier, reducing inflammation and permeability as well as liver damage, as measured by hepatic steatosis and aminotransferase levels [30]. These results, in association with optimal safety and tolerability, make probiotics a promising therapeutic tool in pediatric NAFLD. However, to confirm these results, further larger randomized studies are still needed.

11.4.2 Novel Therapies

Bile Acid. Bile acids are synthesized in hepatocytes from cholesterol through enzymatic pathways and then conjugated with glycine or taurine before secretion into bile and release into the small intestine. In the small intestine, conjugated bile acids are not only involved in lipid absorption and transport but have also been increasingly recognized to function as nuclear receptor binders and to have a role in function of microbiota. Bacteria within the intestine can also chemically modify bile acids and thereby alter the composition of the bile acids [31]. Besides the classic role as detergents to facilitate fat absorption, bile acids have also been recognized as important cell signaling molecules regulating lipid and carbohydrate metabolism and inflammatory response. These molecular functions are mediated through their binding and activation of the nuclear hormone receptor, farnesoid X receptor (FXR), and the G-protein-coupled cell surface receptor TGR5. Intestinal FXR activity upregulates endocrine FGF19 expression, which inhibits hepatic bile acid synthesis via CYP7A1 signaling [32]. Recently, it was showed that hepatic FXR protein content and plasma FGF19 concentrations in children and adolescents with NASH were decreased compared to levels in children with "simple" fatty liver. Hepatic FXR protein level was positively correlated with serum FGF-19 concentrations, and both FXR and FGF19 concentration were inversely and independently associated with NASH [18].

When activated, FXR migrates into the cell nucleus and modulates the transcription of specific genes involved in the regulation of inflammation and glucose and lipid metabolism. Numerous studies, mostly based on animal models, have shown that the use of FXR agonists (e.g., obeticholic acid) could improve hepatic steatosis and steatohepatitis [33].

Recently, ursodeoxycholic acid (UDCA) has been supposed to be involved in several other mechanisms, such as glutathione synthesis and activation of glucocorticoid receptor, contributing to the antioxidants anti-inflammatory pathways. Despite these effects, administration of UDCA does not result in concrete benefits in

treating NAFLD [34]. In contrast, the FLINT study, conducted in an adult population with NASH, showed that treatment with obeticholic acid (6-ethylchenodeoxycholic acid), FXR agonist, was effective in about half of the treated patients. Obeticholic acid improved the biochemical and histological characteristics of NASH compared to placebo. All histological parameters of NASH (steatosis, hepatocellular swelling, and lobular inflammation) and fibrosis have improved. The improvement of fibrosis, although limited, demonstrated that this therapy could be useful in preventing progression to cirrhosis [35]. In pediatric age, this bile acid has not yet been tested.

Finally, Liraglutide is an analog of glucagone-like peptide 1 (GLP-1), a gut-derived incretin hormone that induces weight loss and insulin sensitivity. In 2016, the LEAN study, conducted on 52 adult patients with NASH, showed that liraglutide led to histological resolution of steatohepatitis. The treatment was safe and well tolerated by the patients, but today no information is available about liraglutide in pediatric setting [36].

Many other trials must be done to understand the therapeutic role of these molecules in NAFLD, mainly in pediatric population.

Bariatric surgery. Bariatric surgery and non-surgical obesity treatments based on minimally invasive intragastric balloons are emerging as therapeutic alternatives that should be carefully considered in obese children with NAFLD, mostly in patients with numerous, unsuccessful weight loss attempts [37]. In 2015, a position paper of ESPGHAN has established eligibility criteria for bariatric surgery in pediatric patients: selected obese patients with BMI >40 kg/m^2 and severe comorbidities (including NASH with advanced fibrosis) or with BMI more than 50 kg/m^2 and mild comorbidities (including NASH) [38].

In a recent pediatric trial, laparoscopic sleeve gastrectomy, a restrictive intervention consisting in the removal of the gastric fundus, has proved to be more effective than lifestyle approach, even when combined with intragastric devices, in reducing NASH and fibrosis in obese patients after 1 year of treatment and in improving dyslipidemia, sleep apnea, and hypertension [39].

A novel technique used in children is the adjustable gastric banding (AGB). The banding operation is characterized by an externally compressive device on the upper portion of the stomach, which can be inflated or deflated with a subcutaneous port, permitting adjustment of the degree of gastric compression to limit stomach distention and food intake. The main benefit of this technique is to be completely reversible, with discrete efficacy pales in comparison with other bariatric operations. The weight loss response with AGB is highly variable, and prospective studies in adults show a body weight loss of about 20%. Currently the trials are underway in adolescent populations [40].

Bariatric surgery should not be considered as a first-line therapy and a careful evaluation of the patient, considering emotional, psychological, and clinical features, should be performed before performing surgery. Moreover, further studies are needed in order to evaluate long-term efficacy and safety of these procedures (Fig. 11.1).

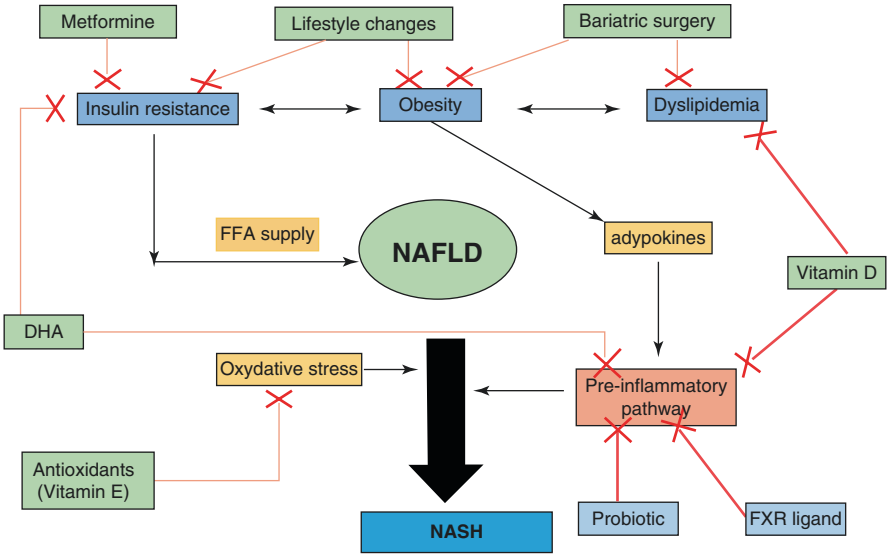

Fig. 11.1 Therapeutic targets of pediatric NAFLD

11.5 Conclusion

NAFLD is often perceived by the patients as a "minor" disease compared to other liver conditions, but recent studies stated that fibrotic potential of NAFLD is as severe as that of chronic hepatitis C, with an average interval time of transition from NASH to cirrhosis estimated around 8–10 years [41]. Prevalence data have decreed NAFLD as the most widespread liver disease in Western countries. 30–40% and 3–5% of adult US population is affected by NAFLD and NASH, respectively: this reflects the risk for millions of people to develop, over the years, end-stage liver disease potentially requiring liver transplantation (LT). Over the past 25 years, in the USA, the number of LTs performed for NASH cirrhosis has doubled from 5.5 to 11% of all reported LTs, and to date NAFLD is the third cause of LT preceded only by alcoholic liver disease and hepatitis C virus. Considering the prevalence trend of NAFLD, the delay of diagnosis due to the absence of non-invasive diagnostic tool, the absence of effective treatments, NASH cirrhosis is expected to become the main indication for LT by 2030 [42].

Because of its natural history, LT for NASH cirrhosis is a rare occurrence in the pediatric context, in fact the average age of transplantation is around 58.5 ± 8 years old [43]. However, some reports of severe hepatic disease and HCC being to be described now also in adolescents and young adults. Moreover, it is important to consider that the worldwide prevalence of NAFLD in children is a worrying phenomenon because this disease is closely associated with the development of both

cirrhosis and cardiometabolic syndrome in adulthood. Therefore, the identification of early disease markers and prompt therapeutic approach represents important objectives of the research programs in this field in the next decades.

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12 Diagnostic Algorithm for the Identification of NAFLD in Primary Care

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Abbreviations

AASLD	American Association for the study of liver diseases
APASL	The Asian Pacific Association for the Study of the Liver
EASL	European Association for the Study of the Liver
GP	General Practitioner
MetS	Metabolic syndrome
NAFLD FS	NAFLD fibrosis score
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
T2DM	Type 2-diabetes mellitus
TE	Transitory elastography

Nonalcoholic fatty liver disease (NAFLD) is extremely frequent. According to Younossi et al., about 25% of the world population has NAFLD [1], with large variations among continents and countries in prevalence, with Western countries having the highest prevalence and African countries the lowest. However, among the large number of individuals with NAFLD only a minority has severe liver disease. This was well demonstrated in an Asian study, where among 922 subjects, NAFLD, based on proton magnetic resonance spectroscopy, was observed in 27.3%, while advanced fibrosis, based on liver stiffness measurement, was found in only 3.7% [2].

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The majority of NAFLD patients are probably being followed in primary care and only a minority is referred to specialist care. In a large study from UK, it was found that NAFLD is the commonest cause of incidental LFT abnormalities in primary care (26.4%), of whom 7.6% have advanced fibrosis as calculated by the NAFLD fibrosis score (NFS) [3].

In this chapter, it will be discussed to which extent the identification and referral of these patients is justified, and what methodology is more useful to achieve it.

12.1 Relevance and Screening for NAFLD in Primary Care

A major point of controversy relates to the need of population screening for NAFLD. According to Wilson and Jungner classic screening criteria (WHO 1968), one of the major requirements for public health screening to be accepted is that there should be an accepted treatment for patients with the recognized disease [4]. In fact, the absence of an effective treatment is probably the major factor against screening for NAFLD. Nonetheless, there is recent evidence of the impressive effects of life-style interventions accompanied by significant weight loss in the improvement or resolution of steatosis/steatohepatitis [5, 6]. Consequently, there may be a role for screening, in order to recommend these lifestyle changes that may simultaneously reduce the increased risk of cardiovascular disease as well as cancers in these patients.

There is general agreement from the Hepatology scientific societies that the screening of the general population is not cost-effective [7–9]. There is however disagreement regarding the screening of particular groups. In fact, the European Association for the Study of the Liver (EASL) states that individuals with obesity or metabolic syndrome should be screened for NAFLD by liver enzymes and/or ultrasound as part of their routine workup [7]. The same guidelines indicate that high-risk individuals, such as those older than 50 years, with type 2 diabetes mellitus (T2DM) or metabolic syndrome (MetS), should undergo evaluation for the presence of advanced liver disease (nonalcoholic steatohepatitis—NASH) [7]. On the other hand, the 2018 American Association for the study of liver diseases (AASLD) guidelines consider that even in high-risk groups from primary care, diabetes or obesity clinics, routine screening for NAFLD is not advisable, mostly due to the doubts regarding the treatment options and diagnostic tests as well as the lack of knowledge regarding long-term benefits and cost-effectiveness [8]. However, it is admitted that there should be a high index of suspicion for the presence of NAFLD and NASH in T2DM, and that it may be useful to identify those that are at low- or high risk for advanced fibrosis [8].

The Asian Pacific Association for the Study of the Liver (APASL) Working Party NAFLD 2017 guidelines suggest that screening for NAFLD may be considered in risk groups such as patients with T2DM and obesity, mostly reasoning that in these subgroups the probability of having severe disease is much higher that in those with NAFLD from the general population [9].

Actually, in primary care, it was found that in patients with T2DM, the prevalence of NAFLD (defined as MRI-PDFF ≥5%) and advanced fibrosis (defined as MRE ≥3.6 kPa) was 65% and 7.1%, respectively [10]. In another study, also in T2DM, and using transitory elastography (TE), among 1918 patients, the proportion of patients with NAFLD and those with advanced fibrosis was 73% and 18%, respectively [11]. Also, among obese subjects the prevalence of NAFLD, based on proton magnetic resonance spectroscopy, and advanced fibrosis, based on liver stiffness measurement, was 61% and 19%, respectively [12].

Nonetheless, a cost-effective analysis using a Markov model found screening for NASH in individuals with diabetes although improving liver-related outcomes was not cost-effective, due to side effects of therapy [13].

12.2 Methods for NAFLD Identification

As shown in the previous section, there is no role for actively searching for NAFLD in the general population.

However, there is evidence of lack of recognition of NAFLD among general practitioners (GPs) in UK, as in the USA [14, 15]. In fact, Blais et al. found that the majority of patients in a primary care unit were not being recognized and evaluated, although all the patients identified had evidence of the metabolic syndrome. Only 3% of patients at a high risk of advanced fibrosis had been referred to a specialist consultation [15]. There seems to exist a gap in the GPs knowledge and cognizance of relevant practice guidelines. It has been reported in one study from the USA that over 40% of GP surveyed were not familiar with clinical published guidelines related with NAFLD management [16]. In general, GPs tend to give excessive attention to elevated aminotransferases while disregarding well-accepted predictors of more serious disease, such as older age, elevated body mass index (BMI), presence of diabetes, hypertension, or hypertriglyceridemia [17], thus overlooking the patients who are in fact in higher risk of more serious disease [15].

Furthermore, it was found that a small percentage of these patients with NAFLD, and no clinical manifestations, had advanced fibrosis, and would benefit from screening for life-threatening complications such as hepatocellular carcinoma or variceal bleeding.

In patients presenting with significant metabolic risk factors such as the existence of diabetes or obesity, in order to identify the presence of NAFLD the best method is liver ultrasound (US). In fact, sensitivity and specificity of ultrasound was found as good as other imaging techniques, such as computed tomography (CT) or magnetic resonance imaging (MRI) [18]. Since US has a low cost and it is safe and accessible, it is likely the imaging technique of choice for screening for fatty liver in clinical and population settings [18]. Furthermore, it can simultaneously evaluate the presence of biliary disease or liver metastases. The major drawback of liver ultrasound is that it does not quantify the degree of fibrosis that is the major predictor of end-stage liver disease, hepatocellular carcinoma, and survival [19, 20].

Table 12.1 Other causes of steatosis

Concomitant or alternative causes of steatosis	Tests for evaluation
Excessive alcohol consumption	History of alcohol consumption >20 g/day—females History of alcohol consumption >30 g/day—males
Drugs history of drug exposure	Tamoxifen, valproate, oestrogens, corticosteroids, tetracycline, amiodarone, perhexiline maleate, methotrexate, chloroquine, L-asparaginase
Exposure to toxics	History of occupational exposure to hepatotoxins
Malnutrition; Kwashiorkor; Total parenteral nutrition; rapid weight loss; jejuno-ileal bypass, extensive resection of small bowel	Clinical history
Lipodistrophy, hypobetalipoproteinemia	Clinical history; laboratory evaluation
Celiac disease	Anti-tissue transglutaminase antibodies positive

If large samples of the population are to be investigated in relation to the presence of NAFLD, serum tests are preferable, due to cost and accessibility issues. There are several scores that have been validated, such as the fatty liver index (FLI), the SteatoTest and the NAFLD liver fat score. These scores have been shown to predict the presence of steatosis, but not the severity [21].

Frequently, the suspicion of NAFLD arises due to abnormal aminotransferases or the finding of steatosis in an US usually done for another reason. At this point, there is need to exclude other causes of fatty liver, the most important being excessive alcohol consumption, with a careful history of alcohol consumption. It is increasingly frequent the coexistence of excessive alcohol consumption with metabolic factors, contributing to steatosis; however, an alcohol consumption of more that 20 g in women and 30 g in men, by definition, excludes NAFLD as a diagnosis [7].

Several other states as well as steatogenic drugs have been associated with the presence of steatosis, and need to be excluded (Table 12.1).

12.3 Diagnostic Algorithm

After establishing the diagnosis of NAFLD, it is necessary to evaluate the severity of the disease, in order to decide who are the patients that need to be referred to a specialist consultation.

The dichotomy between patients with NAFL, i.e., simple steatosis on histology and NASH, those with the necro-inflammatory histologic picture NAFLD spectrum, no longer seems so significant. In fact, it is increasingly recognized through

long-term follow-up studies that patients with simple steatosis can also progress to advanced liver disease, and that it is the degree of fibrosis the major predictor of progression and mortality [19, 20, 22]. Consequently, it became more relevant to stage liver fibrosis than to identify the presence of NASH [7, 8, 23].

Although the gold standard for the diagnosis and staging of NAFLD is still liver biopsy (LB) [24], there is no role for its use in primary practice. The major point is the identification of those patients that have more severe fibrosis and need referral, leaving the decision of doing liver biopsy for the specialist.

12.3.1 Staging for Liver Fibrosis

There are several methods that can allow us to predict who are the patients with advanced fibrosis (Table 12.2). These methods should be used in a stepwise approach, by first ruling out advanced fibrosis, benefiting from their high negative predictive value for advanced fibrosis.

Evaluation should start the simplest noninvasive methods, such as validated scores that can be easily calculated from regular blood tests and anthropometric and clinical data, direct fibrosis blood tests (commercial), and test for evaluation of liver fibrosis. Liver biopsy should be reserved for patients with suspected advanced fibrosis or for those patients where there is no concordance among results.

12.3.2 Scores: NAFLD Fibrosis Score and Fibrosis Score 4

Both scores perform quite well in excluding severe fibrosis and have been well validated. General practitioners (GP) can use them in their everyday practice since the

Table 12.2 Methods for staging liver fibrosis in NAFLD patients

Method		Advanced fibrosis
Scores (biochemistry ±anthropometry)	NAFLD fibrosis score Fibrosis-4 score (FIB4)	>0.676 [25] >2.676 [26]
Blood test (commercial)	Enhanced liver fibrosis (ELF)	≥10.51 [27]
Measurement of liver stiffness	Transient elastography: FibroScan with M or XL probes Acoustic radiation force impulse elastography (ARFI) Magnetic resonance elastography (MRE)	> 8.7 Kpa [28] >1.4 m/s [29] >3.64 [30]

NAFLD fibrosis score: $-1.675 + 0.037 \times \text{age} + 0.094 \times \text{BMI} + 1.13 \times \text{IFG or diabetes (yes = 1, no = 0)} + 0.99 \times \text{AST/ALT ratio} - 0.013 \times \text{platelet count} - 0.66 \times \text{serum albumin}$

FIB-4: $(\text{age} \times \text{AST}) (\text{platelet count} \times \sqrt{\text{ALT}})$

formulas are based on simple and routine biochemical tests or anthropometric measurements that can be incorporated in the database system or downloaded on line.

NAFLD fibrosis score (NAFLD FS) is probably the most widely used score, with a very good capacity for ruling out advanced fibrosis [25]; however, the performance is poor for the diagnosis of advanced fibrosis, furthermore classifying about 20–58% in the indeterminate area [31].

In what concerns FIB4 index, a value of <1.3 showed a 90% negative predictive value, while a value of FIB≥2.67 had an 80% positive predictive value for significant fibrosis [26].

The use of commercial tests such as the ELF test that combine three direct fibrosis tests (i.e., tissue inhibitor of metalloproteinase 1, procollagen III N-terminal peptide, and hyaluronic acid) has been shown a good performance and considerable diagnostic value for the prediction of histological fibrosis stage [32]. However it carries economic costs and is less accessible to be used by the GP.

Just by using the first two scores, NAFLD FS and FIB4, GP could rule a significant number of NAFLD patients who have scores below the threshold of significant fibrosis. These patients, all the same should undergo counseling for lifestyle interventions concerning dietary measures and physical activity advise. Also, it is very easy to repeat these scores in a 2 or 3 years time, as proposed by EASL guidelines.

Another possibility, if there is availability, is to use methods to evaluate liver stiffness.

12.3.3 Elastography Measurements

12.3.3.1 Transient Elastography (TE), Using FibroScan with M or XL Probes

The use of TE is quite easy and reproducible. It is not a difficult procedure to learn, and can be performed after minimal training (about 100 examinations), by a medical doctor, a nurse or a technician [33]. It can be performed as a point-of-care test, simultaneously estimating the degree of hepatic steatosis and fibrosis, and has shown higher accuracy in diagnosing advanced fibrosis than the above-mentioned fibrosis prediction scores [34–36]. The major problem with the application of TE is that although it has an excellent sensitivity and a high negative predictive value (NPV) [28, 37–41], thus allowing the exclusion of advanced fibrosis, it has a low positive predictive value (PPV), leaving us with a group of patients that need further confirmation of advanced fibrosis. That confirmation can be done by liver biopsy, or another non-invasive test [42]. Recently it was

demonstrated that by repeated measurement of TE in cases of high liver stiffness measurement (LSM), about one-third of cases will not be confirmed as high, increasing the PPV from 45% to 61% and reducing the number of liver biopsies needed [43].

12.3.3.2 Acoustic Radiation Force Impulse Elastography (ARFI)

ARFI is an ultrasound-based technique that is quite cheap, and can be used during the ultrasound procedure. It has been shown accuracy for the diagnosis of fibrosis although it is a technique with some degree of operator dependency [44, 45]. In a head-to-head comparison with magnetic resonance elastography (MRE), it was less accurate to diagnose fibrosis in obese NAFLD patients [46].

12.3.3.3 Magnetic Resonance Elastography (MRE)

Regarding MRE, it has been shown that using a stiffness cutoff of 3.63 Kpa, an AUROC of 0.924 for diagnosing advanced fibrosis is obtained [47]. If, in addition, a three dimensional (3D) MRE is used with a stiffness cutoff of 2.43, then an AUROC of 0.962 for diagnosing advanced fibrosis is obtained [48]. In fact, this technique has the higher accuracy for fibrosis staging in NAFLD [30, 34], but it is not a practical tool for primary care practice, due to accessibility and costs. It should be reserved for referral centers.

There is also the possibility of combining the scores with elastography, as suggested by Cahn et al., using a novel two-step approach [49].

12.4 Summary

In the primary care setting, GPs should have a great level of suspicion for NAFLD, particularly in high-risk groups. As shown in Fig. 12.1, when facing the evidence of steatosis, usually through ultrasound, the next step is ruling out causes such as excessive alcohol consumption or other steatogenic situations or medications. To assess the risk of progressive disease, probability of advanced fibrosis degree can be calculated by the use of combined scores such as NAFLD FS or FIB-4. If the scores are negative, they can be repeated in 2 or 3 years. If the scores are indeterminate or positive, the patient can either be immediately refereed to a specialist consultation, or undergo an elastography method, such as Fibroscan or ARFI, if there is availability.

If the patients with elevated risk of fibrosis are referred precociously, this will be able to decrease the morbidity and mortality of NAFLD-related liver disease.

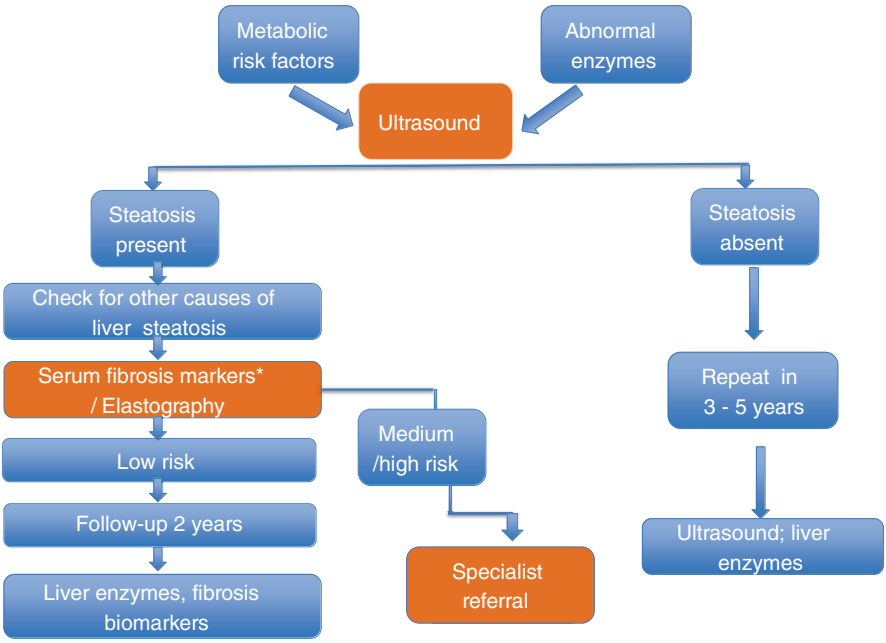

Fig. 12.1 Algorithm for decision in primary care. *Serum fibrosis markers: NAFLD Fibrosis Score, FIB-4, commercial tests (FibroTest, FibroMeter, ELF). (Adapted from NAFLD EASL guidelines [7])

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Non-invasive Diagnostic Approach to NASH: Biological Markers

13

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13.1 Introduction

Traditionally considered the hepatic manifestation of the metabolic syndrome, non-alcoholic fatty liver disease (NAFLD) has dramatically increased in concert with the epidemics of both obesity and type 2 diabetes. A recent meta-analysis estimates that the prevalence of NAFLD is 24.1%, ranging from 13.5% in Africa to 31.7% in Middle-East [1], with some recent general population studies reporting a prevalence higher than 40% [2]. However, these prevalence rates are only expression of a mean data, the prevalence of NAFLD being higher than 60% in at-risk individuals like those older 50 years and with obesity and/or diabetes [2, 3]. Less accurate data also suggest, in general population, a prevalence of non-alcoholic steatohepatitis (NASH)—the progressive from of NAFLD—ranging from 1.5 to 6.4% [1]. All these evidences account for NAFLD becoming the most common cause of chronic liver disease [4], and the growing cause of hepatocellular carcinoma (HCC) [5] and liver transplantation [6]. Furthermore, NAFLD is associated with an increased risk of extrahepatic—mostly cardiovascular and cancer—morbidity and mortality [7]. This pessimistic landscape is further enriched by an analysis forecasting, by using a Markov, the burden of NAFLD and its complications from 2016 to 2030 [8]. This study estimates that NASH prevalence will increase to 15–56%, and that overall mortality, liver mortality and advanced liver disease due to NAFLD will more than double [8].

These data underline as a clinical medical need the availability of instruments that allow to identify patients with NAFLD at risk for liver disease severity and for development of complications. Along this line, ideally, we should dispose of biomarkers able to non-invasively predict (1) the presence of liver disease severity;

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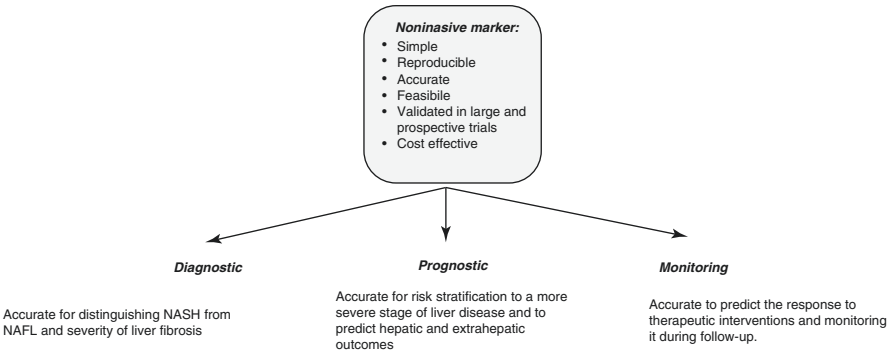

Fig. 13.1 The roles of non-invasive diagnosis. An ideal biomarker should be able to non-invasively predict (1) the presence of liver disease activity and severity; (2) the progression towards a more severe stage of liver disease associated with the occurrence of hepatic complications like HCC and liver decompensation and also the occurrence of extrahepatic complications, and the risk of hepatic and extrahepatic death; and (3) the response to lifestyle correction and/or pharmacological treatments

(2) the progression towards a more severe stage of liver disease, the occurrence of hepatic complications like HCC and liver decompensation, the occurrence of extrahepatic complications and the risk of hepatic and extrahepatic death and (3) the response to lifestyle correction and/or pharmacological treatments (Fig. 13.1).

13.2 Prediction of Liver Disease Severity

Available studies on the natural history of patients with NAFLD clearly demonstrated that the presence of NASH and the development of fibrosis are two clinically relevant disease modifiers able to determine and negatively drive the prognosis of NAFLD patients. NASH can be considered the engine of fibrosis progression in patients with NAFLD. A recent meta-analysis demonstrated that patients with NASH when compared to those with simple steatosis had a faster, double, annual fibrosis progression rate [9]. Consistently some studies reported that the risk of developing cirrhosis and die was higher for NASH patients, even if the strength of this association was attenuated after adjustment for fibrosis stage. On the other hand, two independent retrospective cohort studies from USA and Europe [10, 11] and a meta-analysis [12] pooling all the available evidence on the natural history of NAFLD patients have clearly shown that the severity of liver fibrosis—mostly driven by the underlying NASH—estimated by liver histology is the strongest predictor not only of liver-related complications but also of important extrahepatic diseases, including cardiovascular disease and extrahepatic malignancy. As a consequence, a comprehensive evaluation of liver damage—in particular the discrimination between simple steatosis and NASH and the evaluation of the severity of fibrosis—is a crucial point in the management of patients with NAFLD.

Liver biopsy still remains the "gold standard" procedure for the quantification of the amount of steatosis, and, most relevant, for the distinction between NASH and simple steatosis, and the assessment of the stage of fibrosis [13]. However, it has clear disadvantages such as it is an invasive, painful and costly procedure with potential life-threatening complications for the patient. It is also affected by diagnostic pitfalls like sampling error, potentially under or overestimating liver damage, and intra-observer variability, this issue being less evident for the quantification of fibrosis respect to the diagnosis of NASH [14, 15], where the introduction of the new FLIP histological algorithms should reduce discrepancies among pathologists [16]. In alternative, recent studies suggest magnetic resonance (MR)-based tests like standard for assessment of steatosis, fibrosis, and, perhaps, NASH in patients with NAFLD, these imaging tools being accurate and with the advantage of non-invasively exploring the whole liver [17]. However, lack of full validation, costs and non-availability in clinical practice for a large volume of individuals make these, at least until now, not suitable in clinical practice. Consistent with all the above, great research efforts exist in order to identify non-invasive methods for the assessment of NAFLD severity in terms of NASH and fibrosis.

Before to describe available tools for the non-invasive assessment of NAFLD, it appears very relevant to stress two easy but key issues about this topic, i.e. the role of the use of ALT levels and obesity. Regarding ALT levels, it is well known that roughly 80% of patients with NAFLD have normal ALT serum levels. However, what is crucial is whether ALT alone can discriminate, in NAFLD patients, those with/without liver damage. In an Italian study in 2008 in 458 patients with NAFLD who underwent liver biopsy, NASH was diagnosed in 59% of 63 patients with normal ALT values, the distribution of fibrosis stages being not different according to ALT levels [18]. Consistent with these results in another US study on a smaller sample of 238 patients with NAFLD, 56 with normal ALT, the prevalence of NASH and advanced fibrosis in this last group was 10.7% and 26.8%, respectively [19]. These studies overall suggest that ALT levels alone cannot be used to exclude NAFLD patients with significant liver damage, especially in presence of metabolic risk factors. Similarly, the absence of obesity alone cannot allow us to exclude, in NAFLD, the presence of NASH and/or fibrosis. Large multicentre Italian studies reported a prevalence of NASH of 55% in NAFLD patients without visceral obesity, and a prevalence of NASH and significant fibrosis of about 20% in the so-called lean NAFLD [20], a new identified growing subset of NAFLD patients with a body mass index of <25 kg/m^2. All these data suggest that a singular element cannot be used to rule-in or rule-out the presence of liver damage in NAFLD, but we need of more complex non-invasive methodologies, including instrumental devices, that are not the object of this chapter, and serum markers—i.e. parameters measurable in serum.

13.2.1 Prediction of NASH

The non-invasive identification of NAFLD patients with NASH still represents a relevant unmet medical need.

Different biological markers have been studied and proposed for the non-invasive diagnosis of NASH (Table 13.1), even if CK-18 serum fragment is the most studied marker in this setting. Apoptosis represents one of the most relevant features in the pathogenesis of NASH. Consistent with this evidence, serum levels of CK-18 fragments and total length CK-18 measured by a M30 (cleaved CK-18) and M65 (cleaved + uncleaved CK-18) antibody enzyme-linked immunosorbent assay, respectively, and expression of hepatic apoptosis, have been investigated as potential non-invasive markers of NASH. The first report on a small cohort of 44 NAFLD patients showed that CK-18 fragment levels were significantly higher in patients with NASH compared to individuals with simple fatty liver or to healthy controls [21]. These results have been largely validated in several studies, including a large study from the same research groups and studies conducted in the setting of patients with morbid obesity who underwent bariatric surgery [32–34]. The promising results of CK-18 serum fragments as non-invasive marker of NASH have been also confirmed in a meta-analysis that reported a summary AUC for the prediction of NASH of 0.82 [22]. Other studies also searched for improving the diagnostic accuracy of CK-18 serum fragments by combining it with other variables. Shen and colleagues [24] elaborated a model for the non-invasive diagnosis of NASH and composed by CK-18, adipocyte fatty acid binding protein and fibroblast growth factor 21, reporting a good diagnostic performance. Younossi and colleagues [25] combined CK-18 serum fragments with serum levels of adiponectin and resistin obtaining an accurate prediction of NASH, with an AUC of 0.85. Another attempt to improve the diagnostic accuracy of CK-18 serum fragments has been based by elaborating a panel composed by CK-18 fragments and soluble Fas levels—other marker of apoptosis—by obtaining an AUC of 0.93 in the training and of 0.79 in the validation cohort [26]. CK-18 serum fragments have been also combined with clinical and biochemical variable. Specifically, the Nice Model, elaborated on patients with morbid obesity, was based on CK-18 fragments plus ALT and presence of the metabolic syndrome [27], and reported an AUC of 0.88 and 0.83 in the training and in the validation group, respectively.

Some authors also compared the performance of CK-18 serum fragments by M30, with total (cleaved and uncleaved) CK-18 levels by M65, and expression of both apoptosis and necrosis processes [35]. Notably, the authors found that M65, compared with M30 CK-18, had higher sensitivity and specificity for the diagnosis of NASH, being also able to predict NASH independently of ALT levels [35]. An Asiatic study also compared CK-18 M30 with M65, overall reporting a similar moderate accuracy with an AUC of around 0.7 for predicting NASH. These studies started to reduce the optimism on CK-18 as a good and reliable marker of NASH. Consistently, a recent paper on a large multicentre cohort of more than 400 patients with histological diagnosis of NAFLD showed that CK-18 M30 was not very accurate for diagnosing NASH (AUC 0.65 (95% CI = 0.59–0.71)) showing a sensitivity of 58% and a specificity of 68%, with considerable overlap of CK18 levels between patients with and without NASH, finally making it inadequate as a screening test for NASH [36]. Along this line, another more recent meta-analysis

Table 13.1 Non-invasive prediction of NASH: biomarkers and combined panels

Name of the test	Author, Year	Parameters	Criteria for NASH Diagnosis	Study population	Accuracy
CK-18	Wieckowska et al., 2006 [21]	CK-18 (cut-off 380 U/L)	Kleiner classification	44 NAFLD group 35 healthy age-matched controls	AUC 0.93
	Musso et al., 2011 [22]	CK-18		Meta-analysis of 9 studies assessed CK-18	AUC 0.82 Se 78%, Sp 87%
	Kwok et al., 2014 [23]	CK-18 M30 fragment		Meta-analysis of 11 articles on CK-18 M30	AUC 0.70–0.87 Se 66%, Sp 82%
	Shen et al., 2012 [24]	CK-18, FGF-21	Steatosis, ballooning and intralobular hepatocyte necrosis or moderate fibrosis	146 NAFLD group 74 control group	AUC 0.71–0.73
	Younossi et al., 2008 [25]	CK-18, adiponectin, resistin	Steatosis and at least one among ballooning with lobular inflammation, perisinusoidal fibrosis and Mallory bodies	69—training group 32—validation group	AUC 0.85 (Se 72%, Sp 91%)
	Tamimi et al., 2011 [26]	CK-18, soluble FAS	Brunt classification	95—training group 82—validation group	AUC 0.93 and 0.79 in training and validation group respectively
Nice Model	Anty et al., 2010 [27]	CK-18, ALT, Metabolic syndrome	Kleiner classification	464 morbidly obese patients 310—training group 154—validation group	AUC 0.83–0.88 Se 84%, Sp 86%, PPV 44%, NPV 98%

(continued)

Table 13.1 (continued)

Name of the test	Author, Year	Parameters	Criteria for NASH Diagnosis	Study population	Accuracy
NASH test	Poynard et al., 2006 [28]	Age, sex, height, weight, TG, ALT, AST, cholesterol, GGT, α2macroglobulin, apolipoprotein A1, haptoglobin, total bilirubin	Kleiner classification	160—training group 97—validation group 383—controls	AUC 0.79 (Se 33%, Sp 94%, PPV 66%, NPV 81%)
Index of NASH (ION) score	Otgonsuren et al., 2014 [29]	Waist-to-hip ratio, triglycerides, ALT, HOMA		4458—estimation group 152—validation group	AUC 0.88 ION ≥50 Se 92%, Sp 60%
NASH score	Hyysalo, 2014 [30]	PNPLA3 genotype, AST, fasting insulin	Kleiner classification	296—training group 380—validation group	AUC 0.77–0.76 in training and validation group, respectively
NASH ClinLipMet score	Zhou et al., 2016 [31]	AST, PNPLA3 genotype, fasting insulin, lysophosphatidylcholine 16:0, phosphoethanolamine 40:6, glutamate, isoleucine, glycine	Brunt classification	318 subjects 223—estimation group 95—validation	AUC 0.87

ALT Alanine aminotransferase, *AST* Aspartate aminotransferase, *AUC* area under the receiver-operating curve, *BMI* Body Mass Index, *CK-18* Cytokeratin-18, *FGF-21* Fibroblast Growth Factor-21, *GGT* Gamma-glutamyl transpeptidase, *HOMA* Homeostasis Model assessment, *NASH* non-alcoholic steatohepatitis, *NPV* negative predictive value, *PPV* positive predictive value, *Se* sensitivity, *Sp* specificity, *TG* Triglycerides, *TIMP-1* TIMP metallopeptidase inhibitor 1

reported a pooled sensitivity and specificity of CK-18 M30 for NASH of 66% and 82%, respectively, suggesting that it is not accurate for use in clinical practice [23].

All in all, contrasting results from literature no longer confirming a very good accuracy, the lack of a well-standardized test and of accepted and widely validated diagnostic thresholds, make CK-18 testing as a promising tool, that, however, cannot be recommended in clinical practice.

Another way investigated for the non-invasive diagnosis of NASH is the elaboration of serum panels based on the combination of clinical and biochemical variables, and some time also including pathogenic serum markers of NASH. Overall, a wide number of non-invasive panels for the diagnosis of NASH have been reported, even if the greater proportion of them lack robust validation data, and this issue obviously limits their recommendation in clinical practice.

NASH test is one of the most investigated panel. It combines 13 parameters—age, sex, height, weight, triglycerides, total cholesterol, α2-macroglobulin, apolipoprotein A1, haptoglobin, GGT, ALT, AST and total bilirubin—and in the original study it showed an AUC of 0.79 for the diagnosis of NASH in both the training group of 160 patients, and the validation cohort of 97 NAFLD patients [28]. This score has been also validated in other independent studies, and a meta-analysis pooling the available evidence confirmed its good diagnostic with an AUC for NASH diagnosis of 0.84 [37]. The elevated number of variables included in the score and the lack of availability of some of theme strongly limit its applicability in clinical practice. Otgonsuren and colleagues also proposed the index of NASH (ION) score, obtained by the combination of waist-to-hip ratio, triglycerides, ALT and HOMA [29]. This score, in a cohort of 152 patients with histological diagnosis of NAFLD, showed a good diagnostic accuracy for NASH in terms of AUC (0.88), with an ION score ≥50 having a sensitivity and specificity of 92% and 62%, respectively [29]. However, a recent multicentre Italian study on 292 NAFLD patients did not longer confirm the good performance of ION for NASH, reporting an AUC of 0.68 [38]. Another interesting non-invasive score, the so-called NASH score, has been proposed by Hyssalo and colleagues [30]. This score, the first considering a genetic variable, includes PNPLA3 genotype, AST and fasting insulin; it was elaborated in a cohort of 296 morbid obese Finnish patients who underwent liver biopsy during bariatric surgery, and then validated in a cohort of 380 Italian patients with histological NAFLD [30]. The authors found a good overall AUC for NASH prediction in both training and validation sets (0.77 and 0.76, respectively) [30]. The same authors further updated this score by adding to PNPLA3, insulin and ALT, five variables identified in serum by mass spectroscopy [31]. The authors finally developed the NASH ClinLipMet score that had better accuracy respect to the original NASH score (AUC 0.86 vs 0.78), even if based on variables not easily available in clinical practice [31].

All in all, available data suggest that today, in clinical practice, we do not have easy to use and accurate tools for the non-invasive diagnosis of NASH. For this reason, a great number of new markers and/or panels of markers are under investigation, with a great contribution arising from "omics" approaches.

13.2.2 Prediction of Fibrosis Severity

The non-invasive prediction of fibrosis, due to its clinical relevance and to the lack of reliable tools for identifying patients with NASH, is a debated topic where a great number of evidences are available.

Several non-invasive tools have been developed to evaluate the severity of liver fibrosis in NAFLD patients, including demographic/serum markers and specific fibrosis panels (Table 13.2).

Table 13.2 Main serum and fibrosis biomarkers for the non-invasive assessment of liver fibrosis among patients with non-alcoholic fatty liver disease

Score	Components	Diagnostic endpoints	Overall AUC (single value or range)
Demographic/serum markers			
AST:PLT ratio index (APRI)	AST, PLT (dual cut-offs)	Significant fibrosis Severe fibrosis Cirrhosis	0.56–0.89 0.56–0.90 0.74–0.85
BARD score	BMI, AST/ALT ratio, diabetes	Significant fibrosis Severe fibrosis Cirrhosis	0.60–0-68 0.67–0.90 0.62–0.74
FibroMeter	Glucose, AST, ferritin, PLT, ALT, body weight, age	Significant fibrosis Severe fibrosis Cirrhosis	0.93–0.95 0.92–0.95 0.88–0.94
FIB-4	Age, AST, PLT, ALT (dual cut-offs)	Significant fibrosis Severe fibrosis	0.74–0-75 0.80–0.86
NFS	Age, hyperglycaemia, BMI, PLT, albumin, AST/ALT ratio (dual cut-offs)	Significant fibrosis Severe fibrosis Cirrhosis	0.67–0.90 0.64–0.93 0.80–0-94
Elift	Age, gender, GGT, AST, PLT and prothrombin time	Severe fibrosis Cirrhosis	0.78 0.85
HEPAMET score	Female gender, age, HOMA, diabetes, AST, albumin and PLT	Significant fibrosis Severe fibrosis Cirrhosis	0.78 (0.74–0.82) 0.86(0.82–0.90) 0.92(0.88–0.97)
Specific fibrosis panels			
ELF	PIIINP, hyaluronic acid, TIMP1	F1 F2 significant fibrosis F3 severe fibrosis	0.92 (Sn 88%, Sp 81%) 0.98 (Sn 94%, Sp 93%) 0.99 (Sn 100%, Sp 98%)
FibroTest	GGT, total bilirubin, α-2 macroglobulin, apolipoprotein AI and haptoglobin		0.88 non- binary AUROC
HepaScore	Age, GGT. gender, bilirubin, hyaluronic acid, α-2 macroglobulin	Significant fibrosis Severe fibrosis Cirrhosis	0.72 0.81 0.90

Table 13.2 (continued)

Score	Components	Diagnostic endpoints	Overall AUC (single value or range)
ADAPT	Age, diabetes, PRO-C3, platelet	Severe fibrosis	0.86
NASH F2-F4			
MACK-3 test	AST, HOMA and CK-18 serum fragments	Fibrotic NASH (NAFLD activity score ≥4 and fibrosis F ≥ 2)	0.847

ALT Alanine aminotransferase, *AST* Aspartate aminotransferase, *APRI* AST:PLT ratio index, *AUC* area under the receiver-operating curve, *BMI* Body Mass Index, *CK-18* Cytokeratin-18, *ELF* European Liver Fibrosis, *eLIFT* Easy Liver Fibrosis Test, *GGT* Gamma-glutamyl transpeptidase, *HOMA* Homeostasis Model assessment, *NASH* non-alcoholic steatohepatitis, *NFS* NAFLD Fibrosis Score, *P3NP* amino terminal peptide of procollagen III, *Se* sensitivity, *Sp* specificity, *TG* Triglycerides, *TIMP-1* TIMP metallopeptidase inhibitor 1

Demographic/serum markers do not depict mechanisms leading to fibrogenesis and/or fibrinolysis but reflect risk factors and biological processes associated with the presence of fibrosis. Furthermore, they have the advantage of combining easily available variables, being of consequence easy to apply in clinical practice. Available non-invasive scores in NAFLD for the evaluation of severity of fibrosis are AST-to-PLT ratio (APRI), BARD score, FibroMeter, FIB-4, NFS, and the recently proposed eLIFT and HEPAMET scores. Among theme, the most validated and used in clinical practice are FIB-4 and NFS. The NFS is a panel based on six readily available variables namely age, BMI, AST/ALT ratio, platelet count, hyperglycaemia, albumin and calculated using a pre-defined formula freely available online (http://nafldscore.com). This score has been elaborated using a large multicentre cohort of 733 patients with histological diagnosis of NAFLD [39], and it showed a good accuracy (AUC: 0.84) for the diagnosis of severe fibrosis. The good diagnostic performance of this panel has been validated by different independent studies and by a recent meta-analysis. This last showed that NFS had an AUC for significant fibrosis (11 studies), severe fibrosis (38 studies) and cirrhosis (8 studies) of 0.72, 0.78 and 0.83, respectively [40]. The FIB-4 combines few and simple parameters—age, platelets, ALT and AST—providing a diagnostic accuracy similar to that before reported for NFS, as also confirmed by several reports [41]. The same before quoted recent meta-analysis reported that FIB-4 had an AUC for significant fibrosis (12 studies), severe fibrosis (34 studies) and cirrhosis (8 studies) of 0.75, 0.80 and 0.85, respectively [40]. Both FIB-4 and NFS use two diagnostic thresholds, one for rule-in (2.67 for FIB-4 and 0.676 for NFS) and another for rule-out (1.3 for FIB-4 and −1.455 for NFS) advanced fibrosis. Mcpherson and colleagues, in a large cohort of more than 600 patients with histological diagnosis of NAFLD, reported that the before quoted used thresholds for diagnosing advanced fibrosis work bad in patients older 65 years, finally proposing new cut-offs which improved specificity without adversely affecting sensitivity [42]. BARD and APRI are other very easy to use

non-invasive scores tested in NAFLD patients. However they have less extensive validation, and are characterized by a worse diagnostic accuracy (APRI: AUC 0.70, 0.75 and 0.75 for significant fibrosis, severe fibrosis and cirrhosis; BARD: AUC 0.64, 0.73 and 0.70 for significant fibrosis, severe fibrosis and cirrhosis) respect to FIB-4 and NFS as demonstrated by a meta-analysis [40]. FibroMeter is another non-invasive score evaluated in NAFLD patients especially in French studies. It is composed by age, weight, fasting glucose, AST, ALT, ferritin and platelet, and in a study on a cohort of 235 NAFLD patients, it showed a very good diagnostic accuracy for significant fibrosis, severe fibrosis and cirrhosis, with AUCs of 0.94, 0.93 and 0.90, respectively [43]. The good performance of FibroMeter for severe fibrosis has been then confirmed in an USA NAFLD population where the observed AUC was 0.86 [44].

Major limitations of NFS, FIB-4 and of the other non-invasive scores are the presence of a relevant proportion of patients with false-positive results, and the high proportion—up to around 50%—of patients falling in the uncertainty, not diagnostic area of the test. Consistently, some studies are searching for improving these limitations. Boursier and colleagues, for the diagnosis of advanced fibrosis, recently proposed the eLIFT score, obtained by the combination of age, gender, GGT, AST, platelets and prothrombin time [45]. Notably, it has similar sensitivity when compared with FIB-4, but fewer false-positive results. Finally, preliminary evidence suggests that Hepamet score, a new panel based on female gender, age, HOMA, diabetes, AST, albumin and platelets, has a better accuracy than NFS and FIB-4 for significant and advanced fibrosis as well as for cirrhosis, also reducing the proportion of unclassified patients [46]. However, the promising results from these two studies are worthy to be largely replicated in external studies, and the inclusion of variables not usually evaluated in clinical practice like HOMA could hamper their use.

Serum fibrosis panels look at the turnover of collagen within the liver, and the most tested in NAFLD are Fibrotest, Hepascore and ELF. Fibrotest, based on the combination of serum a2-macroglobulin, apolipoprotein A1, haptoglobin, total bilirubin and GGT, adjusted for age and gender, showed a mean NonBinAUROCs for fibrosis of 0.878 in a large cohort of 600 patients with biopsy-proven NAFLD [47]. Its good performance was also confirmed in another French study on 452 NAFLD patients, where the accuracy of the test in terms of AUC for F2-F4, F3-F4 and F4 fibrosis was 0.75, 0.77 and 0.80, respectively [48]. The same study also explored the ability of Hepascore, composed by age, gender, alpha2-macroglobulin, bilirubin, GGT and hyaluronic acid, in staging fibrosis in NAFLD [48]. Notably, the authors reported an AUC of 0.75, 0.77 and 0.80 for the diagnosis of significant fibrosis, advanced fibrosis and cirrhosis [48]. Another non-invasive panel most used in UK is the ELF score. It is obtained by the combination of N-terminal peptide of procollagen III (P3NP/PIIINP), hyaluronic acid and tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), and in a cohort of 196 patients with histological diagnosis of NAFLD it showed an AUC for severe fibrosis of 0.93 [49]. Finally, a recently published work has dealt with a new fibrosis algorithm called ADAPT resulting from the combination of Age, presence of DiAbetes, PRO-C3 (a marker of type III collagen formation) and plaTelet count. It is accurate in identifying patients

with NAFLD and advanced fibrosis with an AUROC of 0.86 in the derivation and 0.87 in the validation cohort, respectively, and, even if worthy of further validation, is superior to the existing fibrosis scores APRI, FIB-4 and NFS [50].

Overall serum fibrosis panels showed a good diagnostic accuracy for staging fibrosis in NAFLD, and similar to that observed with non-invasive scores. However, their cost, because patented markers, limits their wider application in clinical practice.

Finally, as for non-invasive diagnosis of NASH, "omics" studies and new technological approaches could provide novel and more accurate non-invasive scores for a correct staging of fibrosis in NAFLD patients.

13.2.3 Combination Strategies

The diagnostic limitations of all the above-quoted non-invasive scores for the assessment of liver fibrosis in patients with NAFLD suggest to improve the diagnostic accuracy by combining more scores/tools. Consistently, EASL-EASD-EASO European guidelines recommend that "the combination of biomarkers/scores and transient elastography might confer additional diagnostic accuracy and might save a number of diagnostic liver biopsies" [51].

Some studies tested for the association of two non-invasive scores, while the greater proportion of studies testing combination strategies were focused on non-invasive scores and liver stiffness measurement (LSM) by FibroScan (Table 13.3). Guha and colleagues evaluated the diagnostic accuracy of the simultaneous combination of ELF with NFS in 196 patients with NAFLD [49]. The authors demonstrated that ELF plus NFS had the better AUCs for the detection of severe fibrosis (0.98), moderate fibrosis (0.93) and no fibrosis (0.84), allowing to spare 88% of unnecessary liver biopsies when aiming to diagnose severe fibrosis [49]. The combination of FibroMeter with LSM was investigated in 225 NAFLD patients. In this study the combination was always better than FibroMeter alone, while the superiority towards LSM was observed only for the diagnosis of significant fibrosis [52]. Finally, the parallel combination of NFS or FIB-4 with LSM allowed to obtain an accuracy for the diagnosis of advanced cirrhosis of about 40%, as expression of a strong reduction in the rate of diagnostic errors (<3%) but a relevant increase in the proportion of patients who filled in the uncertainty areas and so worthy of liver biopsy [53]. The above-presented studies assessed a parallel combination of non-invasive scores/tools, while other clinical evidences are also available about the application of a serial combination. Boursier and colleagues, in a large cohort of patients with chronic liver diseases due to different etiologies, showed that the application of the eLIFT as first test, than followed, in patients with a score of ≥8, by the simultaneous combination of FibroMeter plus LSM, allowed to achieve a sensitivity of 76.1% for advanced fibrosis and 92.1% for cirrhosis [45]. Finally, the serial combination of NFS or FIB-4 as first tests, followed by the use of LSM only in patients where the non-invasive scores filled in the uncertainty area, provided interesting results. Specifically this strategy accounts for an accuracy of about

Table 13.3 Combination strategies

Author, year	Biomarkers/panel tests combined	Population and methods	Results	Comments
Guha [49]	ELF, NFS	196 patients with NAFLD	Addition of simple markers to the panel improved diagnostic performance with AUCs of 0.98, 0.93, and 0.84 for the detection of severe fibrosis, moderate fibrosis, and no fibrosis, respectively	The clinical utility model showed that 88% of liver biopsies could be potentially avoided for the diagnosis of severe fibrosis using combined panel
Ducancelle [52]	FibroMeter with LSM	225 patients with NAFLD	The combination had significantly higher indices than their constitutive tests (FibroMeter and LSM) alone in all diagnostic targets	FibroMeter superiority towards LSM was observed only for the diagnosis of significant fibrosis
Petta [53]	NFS, FIB-4, LSM	741 patients with NAFLD 1. Parallel combination of NFS or FIB-4 with LSM 2. Serial combination of NFS or FIB-4 as first tests, followed by the use of LSM only in patients where the non-invasive scores filled in the uncertainty area	1. FibroScan + FIB-4 Accuracy 43,1%, Uncertainty area 54.1%, Wrong classification 2.7%, False positive 1.5%, False negative 5.5% FibroScan + NFS Accuracy 39.1%, Uncertainty area 58.2%, Wrong classification 2.6%, False positive 2.1%, False negative 3.8% 2. NFS → FibroScan in NFS (1.455–0.676) Accuracy (76%), Uncertainty area (8%), Wrong classification (15.9%), False positive (10.6%), False negative (27.6%) FIB-4 → FibroScan in FIB-4 (1.30–2.67) Accuracy (77.8%), Uncertainty area (6.4%), Wrong classification (15.7%), False positive (8.1%), False negative (32.7%)	1. Paired combination of LSM or NFS with FIB-4 strongly reduced the likelihood of wrongly classified patients (<3%), at the price of a high uncertainty area and of a low overall accuracy 2. Serial combination of NFS or FIB-4 as first tests, followed by the use of LSM only in patients where the non-invasive scores filled in the uncertainty area, provided an accuracy of about 76–78%, as expression of a rate of wrong classification of about 15% and an uncertainty area ≤8%, better than what observed by using each test alone

Boursier [45]	eLIFT, FibroMeter, LSM	3754 patients with CLD due to different etiologies including 34.2% with NAFLD randomized 2:1 in derivation and validation sets – Diagnostic study – Prognostic study	Diagnostic study: eLIFT-FMVCTE algorithm (first-line eLIFT, second-line FibroMeterVCTE in patients with an eLIFT score ≥8) showed a sensitivity of 76.1% for advanced fibrosis and 92.1% for cirrhosis Prognostic study: patients diagnosed as having no/mild fibrosis by the eLIFT-FMVCTE had an excellent liver-related prognosis	This new algorithm, called the eLIFT-FMVCTE, could identify patients with advanced chronic liver disease who need referral to a specialist, and those with no or mild liver lesions who can remain under the care of their usual physician

AUC area under the receiver-operating curve, *ELF* European Liver Fibrosis, *eLIFT* Easy Liver Fibrosis Test, *LSM* Liver Stiffness Measurement, *NFS* NAFLD Fibrosis Score

76–78%, as expression of a rate of wrong classification of about 15% and an uncertainty area of ≤8%, better than what observed by using each test alone [53]. To date, from a clinical point of view, this last strategy is that suggested by European guidelines, and that should be promoted in clinical practice due to its acceptable accuracy and easy reliability.

13.2.4 Prediction of NASH with F2-F4 Fibrosis

Available non-invasive scores for the assessment of liver damage in NAFLD are mainly focused on fibrosis—especially severe fibrosis—or NASH. However, the greater proportion of clinical trials evaluating the effectiveness of pharmacological treatments in NAFLD targets patients with histological diagnosis of NASH and with significant fibrosis. Consistently, available non-invasive scores/tools cannot be useful to identify this group of patients. For this reason, Boursier and colleagues, in a large cohort of 846 NAFLD patients, split in derivation and validation cohorts, elaborated the so-called MACK-32 test based on the combination of AST, HOMA and CK-18 serum fragments [54] (Table 13.3). The authors showed that this score worked better than BARD, NFS and FIB4 for the diagnosis of fibrotic NASH, reporting in the validation set an AUC of 0.847 [54]. This study overall provides very interesting results, even if lack of validation in external studies, and the need of unusual variables like HOMA and CK-18 serum fragments limits its use in clinical practice.

13.3 Prognostic Markers

An ideal non-invasive marker to be used in NAFLD patients should be able not only to diagnose NASH and severity of fibrosis but also to predict the risk of fibrosis progression, of occurrence of liver-related and/or unrelated events, and finally to stratify the risk of overall and both hepatic and extrahepatic mortality. To date no specific markers have been developed for this specific purpose, even if some data are available from studies that assessed the prognostic value of non-invasive scores designed for staging liver disease severity.

Considering the progression of liver fibrosis in NAFLD patients, this is not a linear process, and slow versus rapid progressor were also identified. However, no data exist about non-invasive markers able to discriminate progressor from their counterpart, neither slow versus rapid progressor. Available studies only identified severity of baseline liver disease and metabolic comorbidities as risk factors. An Asiatic study showed that in 52 patients with NAFLD and who underwent second liver biopsy after 36 months, reduction or increase in BMI and waist circumference were independently associated, respectively, with non-progressive or progressive disease activity and fibrosis [55]. Along this line two Italian studies further underlined the role of metabolic factors in fibrosis progression: in 132 obese patients with NAFLD and with a repeated liver biopsy after a median time of 6.4 years, fibrosis

progression was associated with incident arterial hypertension and insulin resistance by HOMA-IR [56]; in 118 Italian NAFLD patients who underwent second liver biopsy, baseline presence of diabetes was a risk factor for a higher fibrosis progression rate [57]. The role of diabetes as predictor of fibrosis was also confirmed in a cohort of 103 US patients [58]. Notably, also in patients with simple steatosis, two recent studies demonstrated a possibility of evolution towards advanced fibrosis, impairment in obesity and presence of diabetes being the main risk factors [59, 60].

All the above quoted data identified risk factors for fibrosis progression in NAFLD, but do not provide data about long-term outcomes. For this purpose, some studies are available testing the predictive ability for hepatic and extrahepatic outcomes of scores used for fibrosis prediction. In a population study on a cohort of 4083 US individuals from the NHANES study and with an ultrasonographic diagnosis of NAFLD, subjects with a NFS suggestive of advanced liver fibrosis had a higher risk of overall mortality, and also of liver-related and cardiovascular mortality, but not of death due to malignancies and diabetes [61]. When moving from general population to patients with biopsy-proven NAFLD followed at tertiary referral centres, similar results were observed. Angulo and colleagues assessed the ability of non-invasive scores in predicting liver events and overall mortality in a cohort of 320 patients with histological diagnosis of NAFLD [62]. The authors reported that, when considering APRI, BARD, FIB-4 and NFS, this last had the best accuracy to identify patients with NAFLD who are at increased risk for liver-related complications or death, the risk being proportionally higher in those at intermediate and high NFS range compared to those in the low range [62]. Similar results were more recently reported in a French study on a cohort of 360 patients with NAFLD who underwent non-invasive assessment of liver fibrosis by APRI, FIB-4, Hepascore, FibroMeter^{V2G} and FibroScan [48]. Notably, among non-invasive scores, FibroMeterV2G showed the best discriminative ability for the prediction of all-cause mortality; very interestingly, non-invasive fibrosis tests, which have been initially developed for the non-invasive diagnosis of liver fibrosis, showed also good discriminative ability for the prediction of mortality from extrahepatic cause [48]. Consistent with these data, stratification of patients with FibroMeter values expression of different stages of fibrosis, well predicted the risk of overall mortality in NAFLD [48]

All the above-quoted data suggest that, waiting for specific markers of prognosis in NAFLD, the use of available scores stem for fibrosis prediction, can also help to stratify the prognosis of NAFLD patients. Further studies are needed to assess the clinical meaning of dynamic changes over time of fibrosis scores on the prognosis of patients.

13.4 Markers of Response to Treatment

The epidemiological and clinical burden of NAFLD, i.e. the increase in the prevalence of NAFLD and of its complications, make the availability of effective therapeutic strategies as key topics in the research agenda. To date the only strategy

demonstrated clearly effective for the therapy of NASH is weight loss that, when obtained both by lifestyle correction of bariatric surgery, can lead to NASH disappearance and fibrosis improvement in a proportion of patients growing according to the extent of weight loss (no less than 5–7%) [63, 64]. Conversely, to date there are no U.S. FDA and EMA-approved therapies for NASH, even if a number of molecules targeting different pathogenic pathways of NASH are under evaluation in clinical trials showing encouraging results.

In an era in which some drugs for NASH treatment will be available, a relevant need is the availability of non-invasive markers that can help us to identify patients who respond to treatment from nonresponders where add or switch to other strategies. However only few data are available about this topic and many efforts should be done. Regarding response to weight loss, some studies suggested that patients carrying the at-risk PNPLA3 genetic variant are more likely to reduce fatty liver after weight loss. Specifically, two independent studies demonstrated that in patients enrolled in a programme of hypocaloric low-carbohydrate diet, or in a lifestyle modification programme (increased energy expenditure and reduced caloric intake) those carrying the PNPLA3 G allele had a significant higher reduction in fatty liver assessed by magnetic resonance spectroscopy (MRS) [65, 66]. Similar results, again by using MRS, were reported in a cohort of patients who underwent bariatric surgery [67]. These studies however only identify patients at higher likelihood of fatty liver improvement by weight loss, while not providing data about improvement of liver damage. Vilar-Gomez, by analysing data from a trial assessing the impact of weight loss on NASH and fibrosis in patients with histological diagnosis of NAFLD, elaborated a score that after 1 year can predict the likelihood of NASH disappearance [68]. This score, namely NASHRES, takes into account the extent of weight loss, the achievement of normal ALT levels, the presence of diabetes and baseline NAS score at histology [68]. Nevertheless, the good diagnostic accuracy of the present score and the need of further validation in independent cohorts, the main limitation to its use in clinical practice is the need of a liver biopsy at baseline. The same group, by analysing data from the same cohort, also elaborated an algorithm to predict fibrosis improvement after 1 year of lifestyle correction [69]. The authors found that a model including change in HbA1c, platelets and NFS as well as ALT normalization accurately predicted fibrosis improvement, being more accurate than NFS, FIB-4 and APRI alone and generating, at the threshold of $\geq$0.497, positive and negative predictive values of 94% and 91%, respectively [69]. Similarly, change in platelets and NFS as well as ALT normalization predicted fibrosis progression [69].

Some other studies evaluated the ability of serum CK-18 fragments as an instrument to predict response to NASH treatment. Data analysed from two separate clinical trials of pharmacological treatments of adult and paediatric NASH showed that there was a significant decrease in CK-18 serum levels in both adult and paediatric patients who obtained an histologic improvement compared to their counterpart and independently of the received treatment [70]. However, the accuracy of CK18 serum levels in predicting liver damage improvement was not better than reductions in ALT serum levels.

All in all the available data provide preliminary evidence about the possibility to predict histological changes in NAFLD patients. Further efforts are needed about this topic, and, in the hypothesis that predictors could change according to the used therapeutic strategy and the searched outcomes, each trial of investigational drugs should search for specific predictors of response.

13.5 Conclusion

The increase in NAFLD/NASH prevalence and in its complications makes necessary to have non-invasive tools to be used for the management of these patients. Available tools help us in an acceptable manner to identify patients with severe liver fibrosis, even if further efforts should be done for diagnosing NASH, for better stratifying severity of liver fibrosis, and—very relevant—for predicting outcomes (Fig. 13.2). Finally, the availability in the next future of pharmacological therapies for NASH should promote the research for the identification of markers of response that could drive the therapy of patients.

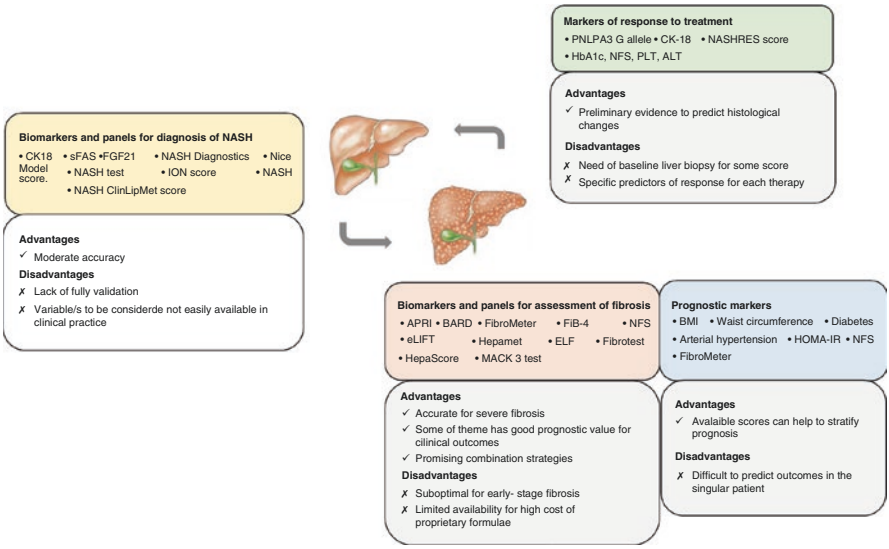

Fig. 13.2 Non-invasive assessment of NAFLD. Available non-invasive tools help us in an acceptable manner to identify patients with severe liver fibrosis, even if further efforts should be done for diagnosing NASH, for better stratifying severity of liver fibrosis, and—very relevant—for predicting outcomes. Finally, the availability in the next future of pharmacological therapies for NASH should promote the research for the identification of markers of response that could drive the therapy of patients. *ALT* alanine aminotransferase, *APRI* AST: platelet ratio index, *BARD score* calculated from BMI, AST:ALT ratio and diabetes mellitus presence, *CK18* cytokeratin 18, *ELF* Enhanced Liver Fibrosis, *eLIFT* Easy Liver Fibrosis Test, *FGF21* fibroblast growth factor 21, *FIB-4* Fibrosis-4 index, *HbA1c* Glycated haemoglobin, *ION score* Index of NASH score, *NFS* NAFLD Fibrosis Score, *PLT* platelets, *sFas* serum levels of apoptosis-mediating surface antigen FAS

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Noninvasive Diagnostic Approach to NASH: Radiological Diagnostics

14

Monica A. Tincopa and Stephen A. Harrison

14.1 Introduction

Imaging has taken on a critical role in the assessment of patients with chronic liver disease due to advancements that allow for more detailed staging and grading of disease. Whereas in the past imaging in patients with chronic liver disease had largely been utilized for assessment of underlying cirrhosis and complications thereof, advances in imaging-based techniques to include capability to provide surrogate quantitative assessments of steatosis and fibrosis have fundamentally changed clinical practice and care patterns in hepatology. These capabilities have become particularly relevant among individuals with nonalcoholic fatty liver disease (NAFLD) given the worldwide prevalence of NAFLD with the need to accurately and noninvasively diagnose and risk-stratify individuals. In particular, there is a critical need to identify and characterize individuals with nonalcoholic steatohepatitis (NASH), the more aggressive phenotype of NAFLD.

Over the past several years, imaging-based modalities to both screen for and quantify degree of hepatic steatosis and fibrosis have become more accurate and accessible. Development of noninvasive diagnostics for NASH poses ongoing challenges as many components of NASH have classically only been assessable via histology. There remains a need for further investigation into imaging techniques that can accurately capture presence and severity of NASH, though several modalities show promise. These imaging biomarkers for NASH would serve a critical role in risk stratification of large at-risk populations and help identify patients at most

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Table 14.1 Benefits and drawbacks of imaging-based techniques to assess for NASH

Imaging modality	Benefits	Drawbacks
VCTE	Cost/accessibility Portability	Concerns about performance: high BMI, severe steatosis, ascites, inflammation, hepatic congestion, alcohol
MRE	Higher diagnostic accuracy	Cost, Accessibility Concerns about performance: severe inflammation, iron overload Claustrophobia
LMS	Higher diagnostic accuracy	Cost, Accessibility Claustrophobia

urgent need of therapy and monitoring. In this chapter, we will review the current state of imaging techniques for the assessment of hepatic steatosis, fibrosis, and NASH, and highlight areas of interest for future work (Table 14.1).

14.2 Radiological Diagnostics: Steatosis

14.2.1 Ultrasound

NAFLD by definition requires the presences of fat deposition in the liver, with a minimum of ≥5% of hepatocytes with steatosis [1]. Conventional ultrasound (CUS) is routinely obtained in clinical practice due to widespread availability, low cost, and tolerability. Assessment of steatosis is qualitatively inferred based on brightness of sonographic images of the liver compared to adjacent structures [2]. In addition to this dichotomous approach, overall degree of steatosis can also be categorically assessed (mild, moderate, or severe). The primary limitation of conventional US stems from the qualitative nature of these assessments that result in lower overall sensitivity, accuracy, and reproducibility [3]. In general, 20–33% steatosis is thought to be the level at which CUS can reliably detect steatosis for screening purposes with a sensitivity of approximately 80% and a specificity of 86% [4, 5]. Performance characteristics of CUS are particularly relevant in obese subjects where quality of images may be affected [6, 7]. Quantitative US (QUS) has been evaluated in a limited number of studies, and may offer superior diagnostic accuracy [8]. QUS uses additional acoustic parameters including backscatter coefficient to characterize tissue microstructure, and in comparison to MRI–proton density fat fraction (PDFF), it has had an AUROC of up to 0.98 [8].

The controlled attenuation parameter (CAP) measurement is an US-based method to assess degree of hepatic steatosis as part of vibration controlled transient elastography (VCTE) systems. During VCTE, a 3.5 MHz signal (M probe) or 2.5 MHz (XL probe) is emitted and a return wave is graded in dB/m. Similar to other US-based assessments of steatosis, there are concerns regarding reliability of CAP assessments. A meta-analysis of 2735 patients with various causes of liver disease noted an area under the receiver operating characteristic curve (AUROC) of

0.82 for presence of steatosis compared to liver biopsy [9]. There has been concern regarding impact of probe type (XL vs M probe) as it relates to CAP measurement, with XL probes potentially overestimating measurements [10, 11]. The optimal cut-offs for different degrees of steatosis are unclear, though a level of 288–302 db/s has been cited as a consideration for detection of 5% steatosis and 337 db/s for S ≥ 3 [12, 13].

14.2.2 CT

CT is an infrequently used modality to assess for NAFLD in large part due to radiation associated with this modality, but individuals obtain CT scans for other indications as part of their medical care and thus CT imaging can be used to assess for steatosis. In general, CT is thought to be more specific to assess for hepatic steatosis than US. In unenhanced CT scans, attenuation values are used to evaluate hepatic triglyceride (TG) content as reduced attenuation has been correlated with amount of intrahepatic steatosis [14]. Using this approach, either to total attenuation value in Hounsfield units (HU), HU difference between the liver and spleen or ratio of liver to spleen HU is used. Depending on the HU cutoff chosen, non-contrast CT has reported sensitivity and specificity for hepatic steatosis of ≥30% between 73%–100% and 95%–100%, respectively [15]. The addition of contrast to CT evaluations can impact the sensitivity and specificity for hepatic steatosis evaluation, primarily due to variability in contrast protocols and alterations in perfusion.

14.2.3 MRI

There are several MRI-based methods to quantify hepatic steatosis and in general, MRI-based imaging is thought to have the highest diagnostic accuracy. The two primary MRI-based methods include MR spectroscopy (MRS) and the MRI-based proton density fat fraction (PDFF). MRS noninvasively measures proton signals as a function of their resonance frequency. These signal intensities correspond to specific frequencies of water or fat and can then be quantified into a fat signal fraction. MRS has excellent sensitivity, even for trace amounts of fat [16]. Despite this sensitivity, MRS has not taken on an important role clinically for the evaluation of hepatic steatosis due to several limitations. This includes restricted spatial coverage, potential for sampling error, need for additional equipment and special expertise for administration and interpretation, and the time-consuming nature of the exam [17].

MRI-PDFF assesses the ratio of MR-visible triglyceride (TG) protons to the sum of TG and water protons [18]. This method can correct for T1 decay and R2* and thus theoretically can account for impact from inflammation, edema, and iron overload. With MRI-PDFF, specific regions of interest (ROI) are determined and thus can account of heterogeneity of fat deposition and is more feasible for longitudinal assessments of changes in fat content. Given these benefits, MRI-PDFF is one of

the most readily used methods for assessment of hepatic steatosis [19]. Prior studies have compared US and MRI-PDFF and have demonstrates higher performance characteristics of MRI-PDFF [20–22].

14.3 Radiological Diagnostics: Fibrosis

The majority of imaging-based tests to assess fibrosis have focused on assessment of liver stiffness as a surrogate for fibrosis due to the mechanical alterations in the hepatic parenchyma as a result of progressive fibrosis. Sheer wave elastography techniques apply the concept that the speed of a propagating mechanical sheer wave is mediated by the stiffness of that medium, and thus reflects the underlying degree of hepatic fibrosis [23]. The major caveat in applying this concept stems from the fact that several other factors including inflammation can also impact stiffness measurements and thus can affect reliability of this methodology, particularly among patients with NASH [24–26].

14.3.1 Ultrasound

There are several US-based methods that have been evaluated for the assessment of hepatic fibrosis. They include VCTE, acoustic radiation force impulse (ARFI), and sheer wave elastography (SWE). ARFI and SWE are of interest, given that it can be integrated into a CUS device. Among these three methods, the most data exist for VCTE in terms of performance characteristics within NAFLD and NASH. Overall, there is evidence in support of higher reliability of ARFI and SWE compared to VCTE to estimate fibrosis, but further studies are necessary to validate these findings. A single study of 172 patients with NAFLD assessed the AUROC for detection of advanced fibrosis using ARFI to be 0.90 [27]. In this study, BMI did not appear to impact ARFI assessments. This was further supported by results of a meta-analysis of ARFI to detect advanced fibrosis in NAFLD that found it to have a moderate degree of accuracy with a sensitivity of 80.2%, specificity of 85.2%, and an AUROC of 0.89 [28]. While there have been very limited studies comparing ARFI and SWE to VCTE, the existing data has suggested equivalent performance to detect advanced fibrosis with AUROC of 0.84, 0.89, and 0.86, respectively [29].

Overall, these US-based elastography methods have the highest performance for accurate assessment of advanced fibrosis or cirrhosis, with diminishing performance distinguishing across lower stages of fibrosis. Prior studies have reported the AUROC for VCTE to diagnose ≥F3 as 0.75–0.93. Overall, these US-based methods have several advantages for use including lower cost, time efficiency, portability, and comparatively lower cost. The primary disadvantage of this approach stems from concern about technical failure or unreliability of results among patients with very high BMIs, significant steatosis, ascites, and significant inflammation [30, 31]. In order to address some of the concerns related to performance among patients

with higher BMI, two VCTE probes have been developed. The standard M probe examines wave propagation at 25–65 mm, whereas the XL probe examines wave propagation at 35–75 mm [32]. Prior studies have shown more reliable estimates of fibrosis among morbidly obese patients undergoing VCTE when the XL probe is used [33, 34]. There remains concern about severe steatosis affecting fibrosis assessments, though data has been conflicting [10, 35, 36]. The best performance characteristic for US elastography is the negative predictive value (NPV) with AUROCs of 0.77 (95% CI 0.72–0.82) for F ≥ F2, 0.80 (95% CI 0.75–0.84) for F ≥ F3, and 0.89 (95% CI 0.84–0.93) for F = F4 [13].

14.3.2 MRI

Multiple MRI-based modalities have been investigated for the assessment of hepatic fibrosis [37, 38]. Magnetic resonance elastography (MRE) has been a primary modality of interest for quantitative assessment of hepatic fibrosis. In MRE, shear waves are generated using a vibrating plate placed against the body wall, and these shear waves are imaged using specific MRI sequences. These data are used to generate quantitative cross-sectional images of differential tissue stiffness. Using these cross-sectional images, regions of interest (ROIs) are then selected, and an overall stiffness measure is calculated. Prior meta-analyses have shown that MRE has higher accuracy with lower technical failure compared to US-based elastography with an AUROC of 0.93 to diagnose ≥F3 [39, 40]. MRE has similarly been shown to have superior accuracy to assess earlier stages of fibrosis compared to VCTE (AUROC 0.82 vs 0.67 for stage 1 or more and 0.89 vs 0.87 for stage 2) [21]. This represents the main advantage of MRE as a noninvasive modality for fibrosis assessment, particularly among those with severe obesity (BMI ≥35) [41]. There are presently several limitations precluding widespread use including high cost, need for specific programming and radiology expertise to perform the exam, and lack of portability.

3D MRE technology is thought to be even more promising with higher accuracy in individual stages of fibrosis. 3D MRE has the capacity to image shear wave fields in three dimensions of the entire liver as compared to assessing several ROIs in 2D MRE. Data is still emerging in application of this newer technology, but Loomba et al. had demonstrated higher AUROC to diagnosed advanced fibrosis using 3D MRE at 40 Hz vs 2D MRE at 60 Hz (0.98 vs 0.92) [42]. The main limitation of 3D MRE stems from the significant level of expertise to utilize and interpret this technology.

In addition to MRE, there are several other MRI-based methods to assess hepatic fibrosis. These include proton diffusion metrics, T1 relaxation time mapping, and corrected T1 decay using the multiscan platform [43]. The apparent diffusion coefficient (ADC) has been shown to have a significant relationship to fibrosis stage. The diagnostic performance of ADC has been inferior to elastography methods however [44]. Similarly, T1 relaxation time has also not been shown to be a reliable method to assess hepatic fibrosis.

14.4 Radiological Diagnostics: NASH

Developing imaging-based modalities to noninvasively assess for and grade NASH remains challenging. Prior studies have shown that patients with underlying NASH often have elevated liver stiffness measurements even in the absence of significant fibrosis due to the presence of necroinflammation. Studies have also shown a correlation between aminotransferase levels and liver stiffness measurements, suggesting a potential role for elastography to assess steatohepatitis [24]. This relationship is quite complex due to the variable, interdependent effects of different histologic components of NASH. The interaction between impact of inflammation on liver stiffness measurements based on degree of underlying fibrosis is a primary concern that can significantly impact diagnostic accuracy of imaging-based techniques for NASH [45]. Presently there is limited data based on small cohorts with variable study designs that impact interpretation and comparison across studies (Table 14.2). These variations include up to a 6-month time lag from imaging to biopsy and significant heterogeneity in patient characteristics, namely distribution of advanced fibrosis in each cohort and percentage of individuals with NASH vs simple steatosis.

14.4.1 VCTE and NASH Assessment

Studies evaluating VCTE to diagnose NASH have had highly variable results with AUROCs ranging from 0.35 to 0.80 [20, 21, 46]. In general, VCTE appears to be less accurate in distinguishing NASH from simple steatosis in the setting of NASH with minimal fibrosis. Park et al. evaluated 104 patients with biopsy-proven NAFLD, 73% with NASH, and 80% with stage 1–2 fibrosis. Using a cutoff of 5.60 kPa, the sensitivity was 61%, specificity was 59%, and AUROC was only 0.35 [21]. By contrast, Imajo and colleagues evaluated 142 patients with NAFLD, 76% with NASH and 68% with F0–2 fibrosis and found an AUROC of 0.80 for VCTE to detect NASH [20]. In order to optimize the predictive ability of VCTE data to assess for NASH, VCTE data has been combined with other biomarkers to create composite scores. Lee and coauthors designed a composite scoring system including CAP >250 dB/m, LSM >7 kPa, and ALT >60 IU/L. Using this score, the AUROC for NASH increased to 0.81, though the specificity was suboptimal with 21% of "high-risk" patients incorrectly categorized as having NASH [46]. In a similar fashion, addition of CAP score and CK-18 added modest improvement in the diagnostic accuracy of VCTE for NASH, with an AUROC of 0.82 in the study done by Imajo et al. [20]. Sasso and colleagues evaluated a combination of kPa, CAP, and AST to detect patients with NAS $\geq$4 and F $\geq$ 2. In the derivation cohort (N = 281), the AUROC was 0.83 and this high accuracy was maintained across three heterogenous external validation cohorts (0.84–0.92) [47].

Other US-based elastography methods such as ARFI and SWE have not been extensively evaluated for accuracy to diagnose NASH. Palmeri et al. did find in their study of 172 patients with NAFLD that there was no clear association with ARFI results and histologic inflammation or hepatocyte ballooning [27]. To the

Table 14.2 Performance characteristics of imaging-based assessments for NASH

Study	Design (*N*)	% NASH	% Advanced fibrosis[a]	Cutoff	Performance characteristics		
				kPa	AUROC	Sensitivity	Specificity
VCTE							
Imajo (2016)	(142)	76%	32%		0.80		
Lee (2016)							
Park (2017)	(104)	73%	20%	5.6	0.35	61%	59%
MRI							
Chen (2011)	Retrospective, 2D MRE (58)	62%	19%	2.74 2.9	0.93	94% 83%	73% 82%
Loomba (2014)	Prospective 2D MRE (117)	91%	19%	3.26	0.73	42%	92%
Loomba (2016)	Prospective, 2 and 3D MRE (100)	87%	15%	1.92 (2D) 2.42 (3D 60 Hz) 1.93 (3D 40 Hz)	0.75 0.76 0.74		
Gallego-Duran (2016)							
Imajo (2016)	2D MRE (142)	76%	36%		0.81		
Park (2017)	(104)	73%	20%	2.53	0.70	64%	59%
Allen (2017)							
Eddowes (2018)	LMS (50)				0.69		
Mckay (2018)	LMS (77)				0.89		
Noureddin (2018)	MRE + MRI-PDFF (99)			2.795	0.80	0.74	0.81

[a]Advanced fibrosis defined as F3 or F4

contrary, Braticevici et al. assessed ARFI in 71 patients with biopsy-proven NASFL and noted an AUROC of 0.87 (0.78 = 0.95), sensitivity of 76%, and specificity of 83% to detect NASH [48].

14.4.2 MRE and NASH Assessment

Both VCTE and MRE have been evaluated to differentiate NASH from isolated hepatic steatosis. Several studies have evaluated the diagnostic accuracy of 2D MRE for NASH. The associated AUROCs of these studies have been highly variable, ranging from 0.70 to 0.93 [20, 21, 42, 45, 49]. The highest AUROC of 0.93 with a sensitivity of 94% and specificity of 73% when using a threshold of 2.74 kPa was reported by Chen et al. in their retrospective study of 58 patients [45]. Main limitations of that study that potentially may account for discrepancy in other reported AUROCs was the lack standardized histologic diagnosis of NASH and a period of up to 90 days between MRE and liver biopsy. Imajo et al. reported the next highest AUROC of 2D MRE to assess for the presence of NASH at 0.81. In this study, 142 NAFLD patients, 32% of whom had stage 3–4 fibrosis and 76% of whom had NASH histology underwent 2D MRE [20]. Addition of PDFF results and CK-18 levels to the 2D MRE data increased the AUROC minimally to 0.82. The remaining studies using 2D MRE reported AUROC between 0.70 and 0.75 to diagnose NASH. One study evaluated application of 3D MRE at both 40 Hz and 60 Hz to evaluate for NASH with AUROCs of 0.74 and 0.76, respectively, comparable to results reported for use of 2D MRE in that study (AUROC 0.75) [42].

14.4.3 Multiparametric MRI and NASH Assessment

Multiparametric MRI/MRE has been perhaps the most promising methodology being investigated to assess for presence and severity of NASH [38, 43, 50–53]. Multiparametric MRI is a non-elastography-based approach that can quantitatively assess degree of hepatic inflammation and fibrosis through application of T1 mapping and damping ratios [53]. A study of 71 patients with biopsy-proven NAFLD underwent MRI multiscan resulted in an AUROC of 0.8 to diagnose NASH [52]. However, it is important to highlight that in this study, both inflammation and fibrosis increased the corrected T1 measurement and thus caused the differentiation between NASH from fibrosis challenging. The Liver Inflammation and Fibrosis (LIF) score, an MRI-derived quantitative assessment, has been shown to be correlated with hepatic fibrosis and inflammation. The LIF score was used in combination with MRI-PDFF assessment among individuals in the UK Biobank cohort and a US-cohort with liver biopsies and was shown to accurately distinguish between

individuals with and without NAFLD/NASH and among those with F ≥2 and PDFF ≥5% [54]. Another study of 77 patients with biopsy-proven NAFLD combined findings of liver *MultiScan* with fasting glucose to identify patients with NASH with a NAS ≥4 and high-risk NASH (NAS ≥4 and F ≥ 2). Using PDFF alone, the AUROC for NASH was 0.85 (0.77–0.94) and when using cT1 alone, the AUROC for NASH was 0.81 (0.70–0.91). When PDFF, cT1, and fasting glucose were combined, the AUROC was 0.89 (0.81–0.96) for NASH [55].

Multiparametric MRE applies additional mechanical parameters including use of multiple frequencies to help better differentiate inflammation from fibrosis. Gallego-Duran and colleagues evaluated a "NASHMRI" protocol using optical processing methods (E3 harmonic mean, E57 second-order contrast, and E73 averaged mean curvature) applied to conventional non-enhanced MRI to predict NASH [56]. In the estimation cohort, the AUROC was 0.88 and in the validation cohort ($N = 87$), the AUROC was 0.83 with a sensitivity of 87%, specificity of 60%, PPV 71%, and NPV 81%. Allen et al. applied combination of MRI-PDFF, dampening ratio, and complex shear modulus among 83 patients, 37 of whom had NASH with only 3 having F3–4. In this study, it was shown that the damping ratio and shear stiffness correlated with lobular inflammation and hepatocellular ballooning. When combined with PDFF, these three parameters were able to assess for histologic NASH with and AUROC of 0.89, a sensitivity of 68%, specificity of 85%, PPV 0.73, and NPV 0.82 [57]. Data from a multicentre study of 99 patients who underwent MRI-PDFF, MRE, and liver biopsy and had an AUROC of 0.80 [58].

Combination approaches like these may hold the highest ability to detect earlier stages and grades of NASH but will need to be evaluated in larger populations in order to validate their use. A recent study demonstrated cost-effectiveness of multiparametric MRI compared to VCTE, wet biomarkers, and liver biopsy, and demonstrated a cost saving of 150,218 pounds per 1000 patients compared to biopsy to identify patients with NASH [50].

14.5 Conclusion

Current radiological techniques including VCTE, MRI-PDFF, MRE, and multiparametric MRI have good performance characteristics to grade hepatic steatosis and stage hepatic fibrosis. When individually applied to assess for underlying NASH, the performance characteristics become less robust with AUROCs mostly around 0.70–0.80 depending on the prevalence of NASH and advanced fibrosis in each cohort. Combination approaches that incorporate multiparametric MRI imaging protocols with MR-based elastography and serum biomarkers that can capture high-risk features of metabolic syndrome will likely be the highest yield. The primary drawback of this approach remains cost of MRI imaging and lack of accessibility of these highly specialized MRI-based protocols.

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Dietary Approach to NAFLD

15

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15.1 Introduction

Diet and physical activity belong to the key therapeutic options with regard to nonalcoholic fatty liver disease/nonalcoholic steatohepatitis (NAFLD/NASH) [1]. The burden of NAFLD has been dramatically growing in parallel with obesity, diabetes, and outbreaks of metabolic syndrome [2]. NAFLD has become the most common cause of chronic liver disease by representing a risk factor for cirrhosis, hepatocellular carcinoma, and liver transplantation [3], for extrahepatic manifestations such as cardiovascular [4, 5] and kidney disease [6], as well as for extrahepatic malignancies [7]. NAFLD encompasses a wide spectrum of conditions, ranging from simple steatosis to nonalcoholic steatohepatitis, which increases risk of getting cirrhosis and hepatocellular carcinoma. With the growing incidence of obesity, sedentary lifestyles, and unhealthy diet worldwide, an increased prevalence of NAFLD is

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being observed, with Europe witnessing between 20 and 30% of cases [8]. Thereby, it has now been recognized as a major public health problem.

Dietary habits and nutrients are the most important contributing factors to the development, progression, and treatment of nonalcoholic fatty liver disease and the associated metabolic comorbidities. In general, a hypercaloric diet, particularly one rich in trans-fats, saturated fats, cholesterol, and fructose-sweetened drinks, appears to increase visceral adiposity and stimulate lipid accumulation in the liver as well as progression of nonalcoholic steatohepatitis. However, the reduction of calorie intake and supplementation with monounsaturated omega-3 fatty acids [9] have preventive as well as therapeutic effects. In addition, fiber, coffee, green tea, and olive oil could be protective nutrients against NAFLD [10].

Based on the available data, a weight loss of at least 3–5%—achieved by having a hypocaloric diet alone, or in combination with exercise and modifications of lifestyle—generally reduces liver steatosis. However, according to the European Association for the Study of Liver Disease (EASLD) 2012 guidelines, a weight loss of up to 10% is required in order to improve cases of liver necroinflammation. Promrat K. et al. [11] performed one of the most relevant studies where it was found that a loss of at least 7% of body weight, due to changes in diet and lifestyle, improves all of the histological parameters in patients diagnosed with NAFLD. Maintaining a long-term adherence to the diet is an important factor for achieving this objective. In addition, a healthy diet has benefits beyond weight reduction in patients with NAFLD irrespective of whether they are obese or of normal weight [12]. Therefore, nutrition is as an important cornerstone in the prevention and treatment of nonalcoholic fatty liver disease, and patients with NAFLD should receive individualized dietary recommendations.

15.2 Effect of Different Nutrients on NAFLD

15.2.1 Effect of Dietary Fatty Acid Composition on NAFLD

Monounsaturated fatty acids (MUFAs), unlike saturated fatty acids (SFAs), do not worsen insulin sensitivity [13]. For example, MUFAs did not affect insulin sensitivity in the KANWU study [14] or in the more recent 24-week RISCK study [15], in which subjects were randomized to consume either a diet high in SFAs or another diet high in MUFAs. This study concluded that decreased insulin sensitivity was secondary to the content in SFAs but not in MUFAs. Nevertheless, other studies suggest that MUFAs are beneficial in cases of NAFLD and even propose mechanisms. These studies indicate that the effects of MUFAs may be explained by their participation in the regulation of insulin-sensitizing gene expression [16] and in reducing inflammation [17], as well as by their inhibitory effects on nuclear factor kappa B (NF-kappa B) [18]. In another study, MUFAs were shown to reduce the expression of genes related to hepatic lipogenesis, gluconeogenesis, and sterol regulatory-element binding protein (SREBP) in obese rats [19]. Further studies are needed to elucidate the role of MUFAs in NAFLD as well as its optimal dosage.

PUFAs are other fatty acids involved in NAFLD, specially omega-3 fatty acids. Intake of omega-3 supplements leads to improvement in cases of fatty liver. Despite the marked heterogeneity between various studies, omega-3 supplements have been shown, in several clinical trials, and as summarized in a meta-analysis [20], to reduce fat in the liver as measured by ultrasound, MRI, and biopsy. In addition, these lead to improvement in liver enzymes.

15.2.2 Effect of Types of Carbohydrates on NAFLD

It seems clear that excess presence of carbohydrates in a diet increases the level of calories, and this may cause the increase in liver fat content which is found in many comparative studies. However, doubts do exist when one considers the type of carbohydrates. According to existing studies, fructose consumption increases visceral adipose tissue, hypertriglyceridemia, and insulin resistance, which is sufficient to warrant, as a clinical recommendation, a reduction in its consumption for patients with NAFLD. The most common sugar found in fruits is sucrose, while corn syrup (which has a high fructose content) is most common in nonalcoholic beverages. Sucrose consists of 50% fructose and 50% glucose. In a recent study done with healthy subjects, the authors observed an increase in liver enzymes in the subjects consuming 25% of sucrose as part of their total daily intake of calories [21]. In another study, it was found that patients with fatty liver disease consumed fructose syrup twice as much as those without NAFLD (365 kcal vs 170 kcal) [22]. In another study, it was shown that patients who received a high-calorie fructose diet had increased deposition of liver fat as compared to the control group [23]. A four-week, randomized, double-blind, and controlled intervention study [24] showed that the reduction of fructose in the diet of Hispanic-American adolescents with NAFLD caused an improvement in several important factors related to cardiovascular risks, in insulin sensitivity, in C-reactive protein, and in low-density lipoprotein oxidation. However, a recent meta-analysis of 21 interventions concluded that there was lack of enough evidence required to draw conclusions about the effects of fructose or saccharose in nonalcoholic fatty liver disease. Therefore, no evidence was found to recommend the avoidance of their consumption. Thus, the literature review by Chung et al. [25], based on a systematic review and meta-analysis, shows that there is a lack of enough evidence which is necessary to draw conclusions regarding the effects of fructose, as compared with sucrose consumption, in nonalcoholic fatty liver disease. Although a difference between fructose and glucose or the direct role of fructose has not been shown in studies aimed at quantifying the content of liver fat, a study monitoring an isocaloric fructose diet (25% of total daily caloric intake for 10 weeks), as compared to a glucose diet, found that it did impair insulin sensitivity [26].

In spite of studies comparing the effects of macronutrients on liver fat content and insulin have some limitations, we can draw a series of conclusions:

– Hypocaloric diets which are poor in fats and carbohydrates reduce liver fat content, while overfeeding based on hypercaloric diets increases it.
– Low-fat and high-carbohydrate diets, as compared to high-fat and low-carbohydrate diets, appear to decrease liver fat content and improve insulin sensitivity. The deleterious effects of high fat content appear to be due to the presence of SFAs, while MUFAs could be beneficial and recommendable in the diet of patients diagnosed with NAFLD.
– Hypercaloric high-carbohydrate diets increase the liver fat content, but there are no convincing data to conclude that fructose is worse than glucose, even though the metabolism of fructose appears to have comparatively more harmful effects on the liver.

The influence of genetic differences in patients with NAFLD, who maintain different diets, has not been systematically studied.

15.2.3 Fiber and NAFLD

Patients with NAFLD have a poor fiber intake. It has been shown that the mean daily fiber intake of these patients is almost 50% lower than that of healthy people [27]. Recently, Cheng and colleagues demonstrated the relationship between dietary fiber intake and hepatic lipid content in cases of NAFLD. They showed that fiber intake was inversely associated with hepatic fat fraction and intrahepatic lipid [28].

15.2.4 Effects of Micronutrients on NAFLD

Vitamin E. This vitamin acts on oxidative stress and is a free radical eliminator, with an antioxidant action that leads to radical chain reactions such as lipid peroxidation. Vitamin E is thought to act on tissue growth factor TGFß1, peroxisome proliferator-activated receptors (PPAR), apoptosis, and helps in the regulation of involved genes. Lavine et al. [29], in the most extensive clinical trial on pediatric patients, found no significant differences between the placebo and vitamin E groups with regard to the improvement of alanine aminotransferase levels. However, more resolution of NASH was seen in the vitamin E group as compared to the placebo group; this was mainly attributed to the improved ballooning of hepatocytes, but there were no differences in steatosis or lobular inflammation between the two groups. Hasegawa et al. [30] selected 12 adult patients with biopsy-proven NASH and administered vitamin E (300 IU/day) to them for 12 months, observing the improvement in cases of liver inflammation and fibrosis as well as in serum transaminases. Since these initial studies, randomized clinical trials with vitamin E have been performed in the context of NAFLD. A comparison between these trials is difficult due to the variation in selection criteria, different doses of vitamin E, and unclear formulations of vitamin E that may affect its bioavailability. In the clinical trial PIVEN [31], which is the largest documented clinical trial, vitamin E was administered orally, at a dose

of 800 IU/day for 96 weeks, and thereby, it was confirmed that vitamin E has a beneficial effect on patients with NAFLD, through improved serum biochemical indices and favorable changes in the liver biopsy. The long-term effect of vitamin E, as well as its effects on prevention of cirrhosis and long-term survival, remains unestablished. Since some meta-analyses have reported an increase in mortality with high doses of vitamin E [32], attention should be paid to administration of long-term high doses of vitamin E. Furthermore, it has been shown that the addition of another potent antioxidant, such as vitamin C, has not altered the antioxidant effects of vitamin E [33].

Vitamin D. There is epidemiologic evidence indicating that NAFLD and vitamin D deficiency often coexist. The epidemiological data shows that low levels of serum 25(OH)D are associated with NAFLD [34]. The first study to show the association between biopsy-proven NAFLD and vitamin D levels was published by Targher et al., which demonstrated that vitamin D concentrations were lower in subjects diagnosed with NAFLD as compared to matched controls. In addition, vitamin D levels were able to predict the histological severity of NAFLD [35]. In general, the different published studies suggest that patients with NAFLD are more likely to be deficient in vitamins. It seems that the metabolic, anti-inflammatory, and anti-fibrotic properties of vitamin D could be responsible for the possible impact of vitamin D on the progression of NAFLD. Nevertheless, the limitations of the studies (such as different methods used for NAFLD diagnosis, variability in defining vitamin D deficiency, and the employment of different techniques to measure vitamin D levels), the limited number of studies done on human subjects, and the lack of consensus with respect to defining the optimal levels of vitamin D make it premature to recommend vitamin D supplementation for the specific treatment of NAFLD.

Minerals. In general, a progressive deterioration in homeostasis of certain minerals is seen in patients with NAFLD, which may reflect the greater oxidative stress and inflammatory status. In particular, certain minerals such as copper, selenium, and iron have been studied in order to further investigate their possible contribution to the development and treatment of diseases such as NAFLD [36].

It has been observed that high-fructose diets may cause copper deficiency. In rats, fructose consumption alters the metabolism of copper, and copper deficiency may be due to the fact that fructose inhibits its absorption through the intestinal epithelium. In addition, copper deficiency and fructose appear to act synergistically: they accelerate the accumulation of liver fat as well as liver damage [37]. Selenium is a trace mineral that is incorporated into proteins in order to make selenoproteins, which are important antioxidant enzymes. This antioxidant property of selenoproteins helps to prevent cellular damage by free radicals. In this regard, it has been observed in experimental models that selenium supplements cause a decrease in the expression of TGF-β1-induced collagen, IL-8 production, as well as overexpression of antioxidant enzymes [38]. Considering that within both in vitro and in vivo studies, selenium supplements have shown a potential effect on the reduction of oxidative stress, it is important to take into account their potential clinical implications in subjects diagnosed with liver disease. However, a cross-sectional study has shown that increase in the levels of plasma selenium is associated with the elevated

prevalence of NAFLD [39]. Iron has been widely implicated in the pathogenesis of NAFLD and represents a potential target with respect to treatment. For example, hyperferritinemia is generally associated with NAFLD and liver damage, while iron depletion by procedural phlebotomy, in patients with a mild overload of iron, could benefit lifestyle changes by the normalization of liver enzymes and insulin resistance [40]. In addition, iron depletion overregulates glucose uptake, and increases insulin receptor expression and its signaling in hepatocytes within both in vitro and in vivo studies [41], while iron supplements in diet cause dyslipidemia and lead to insulin resistance. However, further studies are needed to evaluate the potential for iron depletion therapy in patients diagnosed with NAFLD.

15.2.5 Other Food Components

Caffeine. Caffeine acts on the signaling pathways which lead to reduction of the activity of the connective tissue growth factor: it is considered to be an important stimulator of liver fibrosis. Many of the cytoprotective antioxidant effects of coffee are thought to be independent from the actual caffeine and are due to other ingredients such as flavonoids and polyphenolic compounds with antioxidant activity. A recent meta-analysis showed that although total caffeine consumption is not related to the prevalence of NAFLD, regular consumption of coffee with caffeine may significantly reduce liver fibrosis in patients with NAFLD [42]. Considering the potential benefits of coffee, we recommend its regular consumption in patients with NAFLD while pointing out that consumption of coffee with caffeine is recommended, but caffeine alone is not. However, the recommended dosage has not been established.

Polyphenols. They form part of a very large family of plant-derived compounds comprising an extensive variety of chemical structures. They are included in many kinds of food items, especially ones with vegetal origins. There exists a considerable amount of evidence indicating the hepatoprotective effects of these biomolecules, unless they are in cultured cells and animal models. The proposed mechanisms of action include reduced fatty acid and triacylglycerol synthesis, increased fatty acid oxidation, and a decrease in oxidative stress and inflammation [43]. To date, their optimal dose and the concomitant length of the treatment period is not known, but the obtained data seem to indicate that nutritional intervention studies could demonstrate their importance in the prevention and treatment of NAFLD.

Prebiotics and probiotics. In recent years, we have seen a growing interest regarding the benefits of prebiotics and probiotics in different diseases and specifically in the NAFLD. Patients with NAFLD have a dysfunctional microbiota [44], and this may promote the progression of NAFLD through rupture of the mucosal barrier of the small intestine and bacterial translocation to the systemic circulation, which leads to systemic inflammation, increased cytokines, and insulin resistance [45].

A recent meta-analysis [46] suggests that probiotics improve transaminases, total cholesterol content, TNF-α, and insulin resistance. Moreover, Wong et al. showed that patients with NASH (demonstrated with biopsy), when treated with probiotics, had significantly lower intrahepatic triglyceride content, waist circumference glucose, and lipid levels [47]. However, there is not enough evidence to recommend use of probiotics in NAFLD. In addition, its possible translation to clinical practice may be limited by the reduced number of studies, the variations in the probiotic strains, the doses, and the variable duration of the intervention period used in the different published studies. However, by virtue of their good safety profile (except in immunosuppressed patients), further studies would be required to analyze their role in NAFLD.

15.3 Food and NAFLD

Macronutrients, micronutrients, bioactive compounds, and other components are part of the food that people eat. In addition, the mechanisms by which certain nutrients may influence the disease are not completely understood. For this reason, rather than nutrients, it is important to analyze the role of food in the pathophysiology and treatment of NAFLD. In the following paragraphs, we will focus on the food items that have been associated with NAFLD in both negative and positive ways.

15.3.1 Meat

In general, high intake of meat is related with glucose intolerance, insulin resistance, and a higher risk of type 2 diabetes [48]. All of these factors are involved in NAFLD pathogenesis. However, a high intake of processed meat has been recently associated with an increased risk of NAFLD [49]. In addition, it has been found that people with NAFLD consume more meat, of all types, than healthy people. The potential explanation for this could be: (1) it has a high level of saturated fats and cholesterol; (2) in many cases, it contains preservatives and additives, and (3) people with high intake of meat usually follow a “Western” dietary pattern.

15.3.2 Fatty Fishes

There are studies that have found associations between a high intake of these fishes and a reduced risk of NAFLD [50]. Allard et al. showed that the total PUFA intake of patients with NASH was below the recommended level [51]. The majority of these fishes (pilchards, sardines, mackerel, trout, salmon, herring, and tuna) are rich in omega-3 PUFAs.

15.3.3 Olive Oil

Most studies, which have been done with rodents, have shown a reduction in total lipid and phospholipid levels and in animals whose diet was supplemented with olive oil, as compared to SFAs [24]. Conversely, Rums et al. [52] observed increased liver steatosis in rats who were overfed with olive oil, as compared with corn oil or echium oil. However, it is important to note that the olive oil group had no evidence of oxidative stress or necrosis, as was shown in the liver biopsies from the other groups. Park et al. [53] showed a downregulation of genes associated with liver lipogenesis and a reduced expression of proinflammatory cytokines, providing information on the mechanism by which olive oil reduces oxidative stress in the liver. Finally, a recent study has shown that mice who were fed with a high-fat diet, rich in extra virgin olive oil, showed a decreased hepatic damage, possibly via an anti-inflammatory effect in adipose tissue, along with modifications in the lipid composition of liver and signaling pathways [54]. Therefore, olive oil may be recommended for patients with NAFLD only when it is consumed as part of a low-fat diet, e.g., a Mediterranean diet pattern. The role of olive oil supplements, as well as their use with other food items, requires further research, specifically to clarify the dose and formulation that may be more effective in the treatment and prevention of fatty liver.

15.3.4 Nuts

They show a great therapeutic potential in the treatment of patients with NAFLD by improving the lipid profile and decreasing liver steatosis and inflammation. However, no randomized clinical trials, aimed at evaluating their role in the histological liver parameters of humans, have been performed. Only longitudinal studies are available which show that consumption of nuts leads to decrease in transaminases within 3 months [55].

15.3.5 Tea

Although the potential protective effect of tea appears to be promising for patients with NAFLD, caution must be exercised because of the cases of hepatotoxicity that have been documented in people who consumed green tea [56]. Despite the recent interest in the antioxidant properties of catechins, which may provide a potential benefit, their good effects on patients with chronic liver disease [57] have not been determined. Although there are epidemiological and experimental data, based on tests in animal models, which demonstrate that tea was likely to mitigate the development or progression of NAFLD, the lack of high-quality clinical trials in humans, at present, means that consumption of tea cannot be specifically recommended for patients with NAFLD.

15.4 Implications of Diet on NAFLD

In almost all consensuses on dietary interventions for patients diagnosed with NAFLD that is associated with obesity, a low-calorie diet is recommended. It is well known that the energy content of the diet is the most important factor that influences liver fat content. Hence, reduction of calorie intake should be recommended to all patients with NAFLD who are overweight or obese [58]. In fact, hypocaloric diets are more effective than changes in diet composition. These data agree with the results of two studies [59, 60], where it was seen that two hypocaloric diets were effective in decreasing the transaminase levels and insulin resistance in obese patients with NAFLD, regardless of the composition of the diet. Calorie restriction, together with physical activity, is the best way to lose weight. A relatively low-calorie diet yields better results as compared to a very low-calorie diet.

Regarding the qualitative composition of the diet, the recommended proportions are as follows: 50–60% carbohydrates and 20–25% lipids. This proportion of carbohydrates in the diet may be considered appropriate to ensure compliance with the nutritional intervention. To achieve these macronutrient recommendations, attention should be also paid to the election of food items. To create a "high-quality healthy diet" that improves cases of liver steatosis, it is particularly important to avoid fructose and trans-fats present in soft drinks and fast food meals, as well as processed food. In addition, consumption of refined grains and fried as well as salty food items should be reduced as much as possible.

The importance of the food items which constitute up a diet has been shown in studies on the DASH (Dietary Approach to Stop Hypertension) diet and the Mediterranean diet.

The DASH diet is rich in fruits, vegetables, and low-fat or nonfat dairy. It also includes whole grains on the most part, along with lean meats, fish, poultry, nuts, and beans as well. It is high in fiber content and low to moderate in fat content. This diet was designed to reduce blood pressure, but it has been observed that it is also beneficial for patients with metabolic disorders, due to the fact that it improves insulin resistance, dyslipidemia, and chronic inflammation [61]. In this context, a recent publication shows that NAFLD patients who followed the diet for 8 weeks reduced their body weight, body mass index, alanine aminotransferase, fasting insulin levels, insulin resistance, triglycerides, and inflammatory markers [62]. The possible mechanisms for the protective role of the DASH diet could be the high intake of antioxidants, fiber, MUFAs, and PUFAs.

Mediterranean diet improves insulin sensitivity and achieves a significant reduction in steatosis (from up to 39 to 7%) with a low-fat and high-carbohydrate diet [63]. It is interesting to note that in this study, these changes were not secondary to weight loss. In addition, compared to similar diets with calorie restriction and low-fat content, adhesion to the Mediterranean diet is associated with improvements in lipid profile and insulin, reduction of ALT, and significant improvement in liver steatosis as determined by ultrasound. In another randomized clinical trial, it was

shown that the Mediterranean diet caused a benefit in cases of liver steatosis and insulin sensitivity, when it was maintained for a period of 12 months [64]. This benefit is postulated to be due to the content of olive oil in the Mediterranean diet, regardless of its calorie content. In a cross-sectional study conducted by our working group [65], it was found that adherence to the Mediterranean diet (assessed by a 14-item questionnaire) was associated with lower grades of steatosis and NASH in patients with NAFLD, as diagnosed through liver biopsy. Meta-analyses which assess the effect of the Mediterranean diet in NAFLD are unavailable, but this dietary pattern has demonstrated its effectiveness in surrogate markers of liver steatosis and insulin resistance. In conclusion, the Mediterranean diet is currently considered to be a healthy eating pattern with respect to many diseases which include metabolic syndrome, cardiovascular disease, and neoplastic diseases. In recent years, an interesting inverse association with NAFLD has been observed, which exalts the Mediterranean dietary pattern as a new therapeutic option for cases of NAFLD. Additional studies are needed to confirm these preliminary data and suggest the employment of a reliable and easy-to-use tool for measuring the benefits of adhesion to the Mediterranean diet in patients diagnosed with NAFLD. In addition, it is important to consider that the Mediterranean diet might be associated with a Mediterranean lifestyle, which could also have beneficial impacts with regard to NAFLD.

15.5 Geometry of Nutrition in NAFLD

Geometric Framework for Nutrition (GFN) is a new methodology of interpreting the ways in which nutrients, other dietary constituents, and their interactions influence physiology and health. Thus, it allows to relate nutrition with health outcomes and to move from molecular to ecological levels [66]. A recent study using GFN in mice shows that nutrient intake has a deep impact on appetite, growth, reproduction, aging, cardio-metabolic outcomes, health, obesity, immune function, and gut microbiota [67]. Thus, GFN could be used to improve the diet in order to get better health outcomes. At the end, it would allow us to design a diet containing precise amount of requisite nutrients/foods. Actually, GFN is being used in studies on humans, with promising results that allow one to focus on the dietary determinants of chronic diseases that are associated with obesity and aging [68].

Up till now, there is only one study, in aged mice, that employs GFN to associate nutrition with NAFLD. The study suggests that a low-protein/high-fat/low-carbohydrate diet increases the probability of having NAFLD. The authors also indicate that the composition of macronutrients may be as important as the energy content of the weight-loss diet. Currently, there are no GFN studies being undertaken on diseases of the human liver. Some pertinent limitations could be the slow progression of liver disease and the need to have liver biopsies in order to monitor the disease.

In the future, GFN will provide a unique tool by which to understand the multiple relationships among nutrition, diet, and health. The concomitant information will not only help to understand the causes of NAFLD but also to define nutritional interventions aimed at prevention and treatment of NAFLD.

15.6 Conclusions

In general, it can be considered that NAFLD is a disease that is caused by an unhealthy diet, which has become the main cause of liver diseases in Western countries. The majority of NAFLD patients follow hypercaloric diets laced with instances of overconsumption of simple carbohydrates and fats (mainly saturated fats), along with reduced intake of dietary fiber and food items rich in omega-3 supplements. The available scientific evidence for dietary and nutritional therapy of NAFLD strongly recommends, with a high quality of evidence, that reduction of body weight through a hypocaloric diet and exercise for a period of 3–12 months can improve liver function and the histology of NAFLD/NASH. A weight loss of 5–10% should be sought along with a 25% reduction in caloric intake of the normal diet for the patients' age and sex, in order to obtain a weekly weight loss of 0.5–1 kg. For improvement of NAFLD/NASH, it is recommended to give priority to energy optimization (lipid restriction) in terms of the proportions of food intake. In addition to calorie restriction, the composition—mainly macronutrients and micronutrients—of the diet and some specific food items may also improve the cases of NAFLD. Although the causality has still not been established, the data reviewed in this document suggest that the consumption of specific food items may modulate the risk of NAFLD and its progression to NASH, along with regulating the risk of other entities including metabolic syndrome. There is enough evidence indicating that patients may benefit from a moderate- to low-carbohydrate (40–45% of total calories) diet, coupled with increased dietary MUFA and n-3 PUFAs, and reduced SFAs. Moderate consumption of coffee, nuts, fatty fishes, and olive oil—all of which constitute a Mediterranean diet—appears to be safe in this context. However, the studies which assess their therapeutic role in patients with NAFLD/NASH are currently insufficient.

While specific recommendations regarding their benefit or dosage are established, patients with NAFLD should be allowed to consume these food items as part of a general diet and physical exercise program.

The importance of adherence to the diet should be emphasized because weight loss and weight maintenance remain a considerable challenge for many individuals and most patients end up regaining weight after an initial weight loss [1].

Future studies are needed on bioactive food compounds, which are able to modulate the activation of the genes involved in lipogenesis, fibrogenesis, lipid peroxidation, and inflammation, thereby representing an attractive therapeutic approach for this condition (Fig. 15.1).

Hypocaloric Mediterranean diet for weight loss and NAFLD/NASH resolution

Early breakfast:

- 1 hypocaloric piece of fruit (avoid bananas, grapes, custard apple). The piece of fruit is preferable to juice.
- 1 skim yogurt or glass of skim milk.
- 1 coffee or tea with skim milk,without sugar.
- Sometimes (2-3 times per week), you could add a couple of biscuits of whole bread or half of a toast of whole bread with extra virgin olive oil (1 tablespoon), or margarine (10 gms), or wholegrain cereals without sugar (30 gms).

Midmorning:

- 1 infusion (tea, chamomile, or mint pennyroyal) or coffee with saccharinc an be taken several times per day, provided that they are taken without sugar.
- 1 hypocaloric piece of fruit, or 1 skim yogurt, or 3/4 nuts.
- Occasionally (1-2 times per week), you could add half of a vegetable sandwich or ham sandwich without cheese.

Lunch:

- 1 salad plate (lettuce, endives, tomato, pepper, onion, asparagus, mushrooms, cucumber, spinach, heart of palm, or little corncob) or cooked/grilled vegetables (cucumber, pepper, cauliflower, broccoli, cabbage, asparagus, mushrooms, spinach, chard, zucchini, eggplants, leek, green beans, beet, carrots, pumpkin, artichokes, potatoes, sprouts, pea, or broad beans with moderation) or vegetable soup.
- Cooked or grilled fish, chicken or turkey (without skin),or beef every other day. Oily fish should be included only once per week.

Fig. 15.1 Hypocaloric Mediterranean diet for weight loss and NAFLD/NASH resolution

- Sometimes (2-3 days per week), instead of fish or meat, you could eat a dish which consists of brown rice, wholegrain pasta, potatoes, and stewed vegetables without fat or sauce.
- Sometimes (2-3 days per week), instead of fish or meat, you could consume a dish made of legumes.

Snacks:

- 1 glass of orange juice (two pieces), or 1 hypocaloric piece of fruit, or 1 skim yogurt, or 3/4 nuts.
- 1 infusion/coffee.

Dinner:

- Vegetable soup, or salad, or cooked or grilled vegetables (which should be different from those consumed during lunch).
- Eggs (omelet or cooked, 2 whites and ½ yolk), or fish, or meat that are cooked or grilled, or seafood with shell or natural tuna.
- Sometimes you could add fresh cheese or Iberico ham without fat.
- Optional fruit·

When eating meat, try to avoid red meat (no more than 1 fillet each for 15 days) and choose white meat (chicken, turkey and rabbit). Water should be the principal drink in all meals. Extra virgin olive oil should be the main source of fat in all meals. The usual ways of cooking should be grilling or steaming in the oven. Avoid sauces.

In summary, total caloric intake should be adjusted to save between 500-1,000 kcal/day.

Mediterranean dietary pattern:

Carbohydrates: 45% to 60% of calories, preferably unrefined

Fiber: 20 to 40 g/day (5 to 15 g of soluble fiber)

Total fat: 20% to 35%.

Fig. 15.1 (continued)

Saturated fat: 7%

Polyunsaturated: 5% to 10%

Monounsaturated: 15% to 20%

Omega-3: preferably through intake of blue fish/nuts, 2 to 4 g/day

Cholesterol: 200 to 300 mg/day

Trans fatty acids: <1% of daily calories

Proteins: 20% of daily calories

Vitamin E: 800 IU/day (supplement)

Daily moderate consumption of coffee

Fig. 15.1 (continued)

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Physical Activity in NAFLD: What and How Much?

16

K. Hallsworth and M. Trenell

16.1 Introduction

Even though physical activity and exercise are recommended as part of treatment for NAFLD [1, 2], there have been no large-scale studies with adequate statistical power to guide health practitioners in prescribing exercise programmes or for generating physical activity guidelines for the management of these patients. Evidence for the benefit of physical activity comes from prospective studies showing that individuals who maintain a physically active lifestyle are less likely to develop insulin resistance (IR), impaired glucose tolerance, or type 2 diabetes [3–6]. Physical activity levels have been shown to be lower in people with NAFLD than their "healthy" counterparts [7–10], and links have been made between low cardiorespiratory fitness and NAFLD severity [11, 12].

Being physically *inactivity* is not just a lack of physical activity, but rather a distinct behaviour in itself, often called "sedentary behaviour." This is becoming a growing problem in the general population [13], and low levels of physical activity are compounded by an increase in physical inactivity. Sedentary behaviour, including activities such as sitting, is reported to be higher in people predisposed to the metabolic syndrome, excessive adiposity, and type 2 diabetes [14–17] and has been shown to be higher in NAFLD [10]. Consequently, increases in sedentary time could play a potential role in the development of or predisposition towards NAFLD, independent of physical activity/exercise and needs to be considered when introducing lifestyle interventions.

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16.2 Physical Activity, Exercise and Metabolic Health

Although much attention has historically been given to the role of nutrition in the management of obesity and NAFLD, emerging evidence suggests that energy expenditure also plays an integral role in adequate metabolic control. Our everyday lives consist of activities which, without us paying conscious effort, have a profound impact upon our health and well-being. Activities related to energy expenditure can typically be broken into four distinct categories throughout the day: (1) sedentary behaviour or inactivity, (2) physical activity, (3) exercise and (4) sleep.

Sedentary behaviour is not simply a lack of physical activity but is a cluster of individual behaviours where sitting or lying is the dominant mode of posture, and energy expenditure is very low. The definition of being sedentary or physically inactive is controversial. Some groups define inactivity as expending less than 1.5 kcal/kg/day in leisure physical activities (National Population Health Survey of Canada: www.hc-sc.gc.ca/fn-an/surveill/nutrition/population/index-eng.php), while the UK National Obesity Forum indicates that 3000–6000 steps/day is sedentary or inactive (www.national obesityforum.org.uk). In the US National Health Interview Survey, adults were classified as sedentary if they did not report any sessions of light to moderate or vigorous leisure-time physical activity of at least 10 min a day (www.cdc.gov/nchs/nhis). Sedentary behaviours are multi-faceted and might include behaviours at work or school, at home, during transport and in leisure time. Typically, key sedentary behaviours include screen time (TV viewing, computer use), motorised transport and sitting.

Physical activity is defined as "any bodily movement produced by contraction of skeletal muscles and resulting in energy expenditure above the basal level" [18] and constitutes many of the activities carried out as part of the daily routine. The term "physical activity" should not be confused with "exercise." Exercise is a subcategory of physical activity in which planned, structured, and repetitive bodily movements are performed to maintain or improve physical fitness. Physical activity includes exercise as well as other activities which involve bodily movement and are done as part of playing, working, active transportation, house chores and recreational activities.

Physical activity can be defined in terms of its metabolic equivalent (MET) level, a physiological measure expressing the energy cost of the task. It is defined as the ratio of metabolic rate (and therefore the rate of energy consumption) during a specific physical activity to a reference metabolic rate, set by convention to 3.5 $mL \cdot O_2 \cdot kg^{-1} \cdot min^{-1}$ or equivalently 1 $kcal \cdot kg^{-1}\ h^{-1}$ or 4.184 $kJ \cdot kg^{-1}\ h^{-1}$ [19]. One MET is considered as the resting metabolic rate (RMR) measured during quiet sitting. Activities of less than 3 METs are classed as "light" (e.g. desk work, watching television, slow walking), 3–6 METs as "moderate" (e.g. walking at 3–4 mph, cycling less than 10 mph), and over 6 METs as "vigorous" (e.g. running, circuit training).

With sleep playing an important role in physiological and cognitive well-being, alongside the large proportion of our lives which is spent asleep, it is not surprising that variations in sleep, whether duration or pattern, influence metabolic and mental

health. Cross-sectional and prospective cohorts reveal that self-reported sleep duration of less than 7 h is associated with an excess risk of cardiovascular disease (up to 33%), type 2 diabetes and all-cause mortality [20, 21].

16.3 Sedentary Behaviour and Metabolic Control

Sedentary behaviour, also referred to as physical *in*activity, holds strong epidemiological, physiological and molecular relationships with the development of over 30 long-term conditions [22]. Subtle changes in sedentary behaviour may contribute to obesity and metabolic disorders, potentially as much as lack of moderate–vigorous physical activity. Both TV sitting (a reliable marker of overall sedentary behaviour) and physical activity are associated with cardio-metabolic health when viewed separately [23, 24] or together [25]. Beyond cardio-metabolic health, 3+ h of daily sitting is linked to all-cause mortality (RR 1.30; 95% CI, 1.06–1.56) [23]. Sedentary behaviour, including activities such as sitting, is reported to be higher in people predisposed to the metabolic syndrome, excessive adiposity and type 2 diabetes [26]. In addition, prospective studies show that a change in TV viewing over 5 years was associated with waist circumference and clustered cardio-metabolic risk score, independent of physical activity [27]. Even if adults meet the public health guideline for leisure-time physical activity, they may have a high risk of becoming overweight or developing metabolic disorders if they spend a large amount of time in sedentary behaviours during the rest of the day [28].

Increasing sedentary behaviour is becoming a growing problem in the general population [13], and low levels of physical activity are compounded by an increase in physical inactivity. One of the seminal studies linking everyday physical inactivity with adverse health showed that people with jobs that involve a lot of sitting (e.g. bus drivers) had double the incidence of cardiovascular disease as those whose jobs include more standing and walking activities (e.g. bus conductors) [29]. The most direct effect of sitting still is that the work performed by the large skeletal muscles in the legs, back and trunk required for upright movement decreases. Sitting for prolonged periods also causes the loss of opportunity for cumulative energy expenditure resulting from the thousands of intermittent muscular contractions throughout the day [30]. Sedentary behaviours involving sitting or lying down are characterised by a low MET value of less than 2, and lower mean daily MET levels are related adversely to metabolic biomarkers and to poorer health outcomes [28]. A recent study by Hallsworth et al. [10] found average daily MET levels were significantly lower in patients with NAFLD when compared to healthy controls.

The majority of the general population are unaware of the potential insidious dangers of sitting too much or the possible benefits of at least maintaining daily low-intensity intermittent non-exercise activity throughout the day. Often, these non-exercise activities occur subconsciously. Energy expenditure of "standing workers" (e.g. shop assistants) was approximately 1400 kcal/day, for work involving some manual labour around 2300 kcal/day, whereas seated workers burned only around 700 kcal/day. More than 90% of the calories burned during all forms of

physical activity were due to this pattern of standing and non-exercise ambulatory movements [30]. The frequency and cumulative duration of non-exercise activity throughout the day is extremely high. People perform intermittent bouts of non-exercise activity throughout most of the day, 7 days/week, 365 days/year. In contrast, the frequency of exercise is more limited, generally to less than 150 min/week. Given the broader opportunities and implications for daily low-intensity activity, it is possible that maintaining this level of activity has greater implications for health and well-being than moderate–vigorous physical activity for those who do not prefer more structured exercise.

Classically, there are three components of human daily energy expenditure (Fig. 16.1): basal metabolic rate (BMR), the thermic effect of food and activity thermogenesis. BMR is the energy required for the core bodily functions and is measured at complete rest while fasted. It accounts for about 60% of daily energy expenditure in a sedentary person. Nearly all of its variability is accounted for by body size, or more precisely lean body mass, with bigger and/or leaner people having a higher BMR. The thermic effect of food is the energy expended in response to a meal and is that associated with digestion, absorption and fuel storage. This accounts for about 10% of daily energy needs and does not vary greatly between people. The remaining component activity thermogenesis can be subdivided into exercise and non-exercise activity thermogenesis (NEAT) which incorporates general, everyday activity. NEAT is the most variable component of human expenditure, and may be the easiest to manipulate for health benefits. NEAT varies between two people of similar size by 2000 kcal/day because of people's different occupations

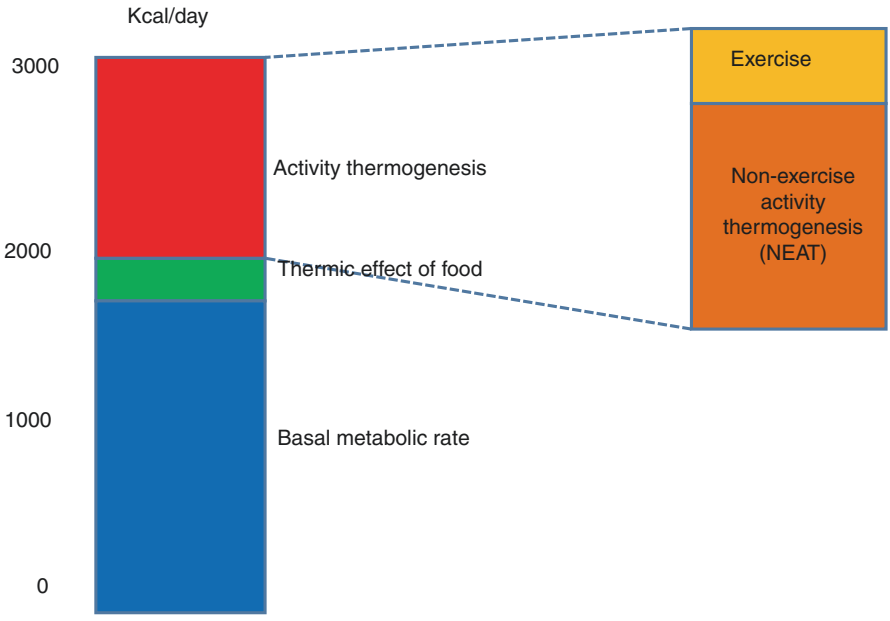

Fig. 16.1 Components of total daily energy expenditure [31]

and leisure-time activities [31]. Occupations that involve physical labour, such as farming, confer higher NEAT values than those that involve more sedentary work. Variability in leisure activities also affects NEAT—those people that choose to sit in the evening watching the television exhibit lower NEAT than those that are out walking the dog. Obesity is associated with low NEAT; obese individuals stand and ambulate for 2.5 h/day less than lean sedentary controls [16]. If we can attempt to address this, either at an individual level by encouraging the person to move more, or at an environmental/societal level by ensuring there are more opportunities to stand/walk throughout the day, then we may have a positive impact on obesity levels and metabolic control.

The links between sedentary behaviour and metabolic health extend beyond the total amount of time spent inactive. Healy et al. [15] report that more interruptions in sedentary time were associated with a decrease in metabolic risk factors. This suggests that it is not only the amount of sedentary time that is important but also the manner in which it is accumulated. As sedentary time comprises a large proportion of waking hours (over 50% for most people—[30]), small changes regarding the interruption of this with regular, short breaks of light-intensity activity could be incorporated across numerous settings and workplaces, increasing NEAT, resulting in beneficial metabolic effects [31]. Regular participation in moderate–vigorous intensity exercise should still be promoted as the predominant physical activity message. However, encouraging a reduction in sedentary time through increasing light-intensity day-to-day activity may be another important public health message for reducing obesity and overall metabolic risk [15, 31]. Encouraging our patients with NAFLD to have regular breaks from sitting throughout the day, especially if they hold a sedentary job, will enhance their daily NEAT levels and increase their calorie expenditure. This is an important therapeutic message to relay to our patients with NAFLD regardless of their disease severity.

Researchers hypothesise that signals harming the body during high levels of physical inactivity are different from those that boost health above normal after exercising regularly [32, 33]. Lipoprotein lipase (LPL) is the first protein directly interacting with and regulating lipoproteins to be studied at the cellular level during physical inactivity. Physical inactivity has a powerful effect on suppressing LPL activity in skeletal muscle, the rate-limiting enzyme for the hydrolysis of triglyceride-rich lipoproteins [34]. Local contractile activity and/or inactivity is the major physiological variable regulating LPL function within the skeletal muscle, and a localised reduction in contractile activity is a potent physiological factor reducing LPL activity. Low LPL function has been linked with blunted triglyceride uptake in skeletal muscle and reduced plasma HDL cholesterol levels.

Increased skeletal muscle LPL has been reported following short-term exercise training [32]. LPL activity was measured in six muscles after intensive training for 2 weeks. Exercise increased LPL activity 2- to 2.5-fold in the least oxidative regions of the leg muscle (fast-twitch white fibres), whereas the most oxidative (slow-twitch red fibres) postural leg muscles that already had high LPL due to non-exercise activity did not display any further increase in LPL after training [30]. LPL activity is generally much greater in the red oxidative muscle types than in the

white glycolytic muscles. By removing the normally high level of postural support by oxidative muscles, this abolished the difference of LPL activity between muscle fibre types. This suggests that the difference in LPL activity between fibre types is primarily due to the level of recruitment in normal daily activity [33] and thus, local changes in metabolism during even light–moderate contractions are the most important physiological stimulus for LPL regulation in skeletal muscle.

There is a growing body of evidence reporting that the majority of people at risk of developing the metabolic syndrome, obesity, NAFLD and type 2 diabetes spend excessive amounts of time inactive and have low levels of NEAT [10, 14–17]. These results are real and applicable to our everyday lives, with one study reporting that with every 1 h increase of television viewing per day that there was a 26% increase in the prevalence of metabolic syndrome [14]. The magnitude of the negative effect of television watching was about the same as the positive health benefit derived from the 30 min of extra physical activity/exercise recommended to improve health. Given the balance between the negative health consequences of physical inactivity and the modest positive effects of exercise in comparison, it is important to identify both activity and sedentary behaviour in developing clinically meaningful interventions.

16.4 Sedentary Behaviour and NAFLD

Increases in sedentary time could play a potential role in the development of NAFLD and, in turn, provide a potential avenue for therapy. Current physical inactivity physiology would suggest that a reduction in LPL activity, as a result of fewer cumulative muscle contractions throughout the day, could predispose to NAFLD through the resultant circulatory hyperlipidaemia. An increase in circulating fatty acids, with fewer being hydrolysed as lipoproteins, will lead to an increased delivery of circulating fatty acids to the liver and hence predisposition to or progression of NAFLD. Increasing circulating fatty acids also exacerbates IR [35] and hyperinsulinaemia which could subsequently increase de novo lipogenesis within the liver. A high level of sedentary behaviour reduces NEAT energy expenditure increasing the risk of a person becoming overweight/obese which is linked to NAFLD predisposition.

Targeting a reversal of sedentary behaviour may provide an additional therapeutic avenue to complement physical activity and exercise guidelines. Decreasing overall sedentary time and increasing breaks throughout the day could be a useful therapeutic message to relay to people with NAFLD, and may be perceived as being more achievable by patients initially than increasing physical activity levels. Any means of increasing NEAT, whether it be at work or during leisure time, may exert positive metabolic benefits. There is limited but promising evidence from prospective cohort studies that identify sedentary behaviour as an independent risk factor for NAFLD [36].

16.5 Physical Activity and Metabolic Control

General health guidelines promote at least 150 min/week of moderate–vigorous leisure-time physical activity or 10,000 steps per day for the primary prevention of cardiovascular disease and decreasing the risks for metabolic diseases [37–39]. However, the majority of people in the general population do not follow this prescription for enough moderate–vigorous exercise, and this may be contributing to the rising numbers of people being affected by obesity, type 2 diabetes and NAFLD.

Evidence for the benefit of physical activity comes from studies showing that individuals who exercise and maintain a physically active lifestyle are less likely to develop IR, impaired glucose tolerance or type 2 diabetes [3–6]. Physical activity appears to result in insulin-receptor upregulation in muscle tissue increasing delivery of glucose and insulin to the muscles, and translocation of GLUT4 to the muscle cell membrane, enhancing non-insulin-dependent glucose uptake [8, 40, 41]. Exercise also has a beneficial effect on NEFA metabolism by enhancing whole-body lipid oxidation [42, 43] and favourably affects overall lipid profile [40, 44], reducing the risk of cardiovascular disease. Physical activity, including exercise, has been shown to improve mitochondrial number and density in skeletal muscle [45]. This results in an increase in oxidative capacity which enhances fat oxidation. Physical activity offers an insulin-independent way of aiding glucose homeostasis in the face of IR and promotes fat oxidation, thus reducing hyperlipidaemia, all of which is key in the prevention and management of metabolic disorders including NAFLD.

16.6 Physical Activity and NAFLD

Physical activity levels are reported to be lower in people with NAFLD than their "healthy" counterparts. A cross-sectional study of Japanese men showed that the prevalence of NAFLD was inversely related to the frequency of self-reported exercise [7]. Those people that exercised for more than 30 min/day on at least 3 days/week were half as likely to have NAFLD as their sedentary counterparts, despite a similar BMI. In a subsequent cross-sectional report, these observations were expanded to state that people without fatty liver engaged in nearly three times more resistance activity than people with NAFLD [8]. Among the NAFLD group, those that engaged in physical activity of any kind or duration had lower fasting serum insulin levels and a lower rate of abdominal obesity even though they had a similar BMI to their inactive counterparts. However, in both of these studies, physical activity levels were obtained from self-reported, non-validated, physical activity questionnaires developed for the purpose of the research, rather than being objectively measured. Perseghin et al. (2007) demonstrated that a higher level of habitual physical activity is associated with a lower level of liver fat and suggested that

this relationship may be due to the effect of exercise per se (n = 191) [9]. Again, this study relied upon self-reporting of physical activity levels rather than using an objective measure, but did use a questionnaire validated for use in the general population. A recent study that used a multi-sensor array to measure activity levels in NAFLD revealed that people with NAFLD spent more time physically inactive and achieved lower levels of physical activity than their healthy counterparts on a day-to-day basis [10]. People with NAFLD not only carried out a lower average level of physical activity but also undertook less moderate and vigorous activity than people without NAFLD. The lower levels of these higher intensity activities may have implications as the intensity of the activity may also play a key role in improving metabolic control. Increasing physical activity levels in people with NAFLD is likely to be of benefit, not only to liver health, but overall metabolic profile, and should be encouraged in a bid to prevent NAFLD progression, the development of type 2 diabetes or cardiovascular disease.

16.7 Exercise and NAFLD

Exercise is one of the cornerstones of NAFLD and NASH management although the evidence underpinning this is still in its infancy compared to other conditions (type 2 diabetes for example). This is likely the product of studies combining diet and exercise interventions until recently. Indeed, in 2012 two independent systematic reviews could only identify a maximum of six studies that had undertaken randomised control trials to explore the effects of exercise on liver fat in people with NAFLD [46, 47]. A more recent systematic review in 2017 was able to identify 24 exercise studies, showing the rapid increase in work in this area [48]. These reviews reveal that exercise, without weight loss, produced a 20–30% relative reduction in intrahepatic lipid.

Different forms of exercise (aerobic, resistance/strength training or high-intensity intermittent training) appear to have similar effects on liver fat [46–49]. More vigorous aerobic exercise does not hold additional benefit for liver fat compared with moderate aerobic exercise [50, 51]. However, it should be noted that all exercise trials are still small and have a wide range of variability in terms of their protocol intensities. The studies to date have been relatively short, lasting in the main between 8 and 12 weeks. Longer-term studies are starting to be published and reveal that if patients continue to exercise for 12 months, the benefits remain [52]. However, if patients do not continue to exercise, the benefits are lost [53]. Moreover, further studies should take into account genetic background of the patients and its influence on response to physical activity. Indeed, PNPLA3 seems to influence response to lifestyle intervention. Patients bearing unfavourable genotype GG did respond better than patients with genotype CC or CG [54].

The mechanisms underlying the change in liver fat following exercise in NAFLD reflect changes in energy balance, circulatory lipids and insulin sensitivity. Much of the early work in exercise in NAFLD has been debated as exercise was accompanied by either dietary changes or diet-induced weight loss leaving the question of

whether there is an exercise-only effect. More recent, better-controlled studies are able to not only demonstrate that there is an exercise-only effect on liver fat but also begin to explore the underlying mechanisms. Exercise has little effect on hepatic insulin sensitivity, but does improve peripheral insulin sensitivity [55] producing a net improvement in insulin action and as a consequence, reducing hepatic de novo lipogenesis. It should be noted that the direct benefits of exercise on glycaemic control are significant, but modest even in people with impaired glucose control [5]. However, tracer studies also show that exercise has a direct effect on lipid flux, with an increase in VLDL clearance contributing to the reduction in liver fat with exercise [56], so not all of the changes in liver fat are attributable to insulin sensitivity alone.

Exercise alone, in the absence of any change in body weight or composition, may enhance insulin sensitivity and glucose homeostasis. Exercise, or muscle contraction per se, provides an insulin-independent way of stimulating glucose uptake from the circulation into skeletal muscle. As the muscle contracts, GLUT4 transporters translocate to the muscle cell wall increasing the capacity for glucose uptake [8]. A larger mass of skeletal muscle, as a consequence of exercise, increases overall glucose storage capacity. Exercise also enhances fatty acid metabolism by enhancing whole-body lipid oxidation [42, 43]. Thus, in people who are IR or have type 2 diabetes, exercise provides a way of improving glycaemic control.

In patients with type 2 diabetes, skeletal muscle mitochondria are reduced in size, and there is reduced activity of the electron transport chain [57]. Mitochondria are normally adaptable organelles and in skeletal muscle in healthy individuals there is considerable plasticity in terms of mitochondrial content, allowing the muscle to adapt to match energy demands of physical activity [45]. Endurance training increases fat oxidation during submaximal exercise. Mild or moderate-intensity exercise (25–65% of VO_2max) is associated with a five- to tenfold increase in fat oxidation above resting amounts because of increased energy requirements of muscle and enhanced fatty acid availability [58]. Several factors contribute to this adaptive response: increased density of the mitochondria in the skeletal muscles, which increases the capacity for fat oxidation; a proliferation of capillaries within skeletal muscle, which enhances fatty acid delivery to muscle; an increase in carnitine transferase, which facilitates fatty acid transport across the mitochondrial membrane, and an increase in fatty acid binding proteins, which regulate myocyte fatty acid transport [58, 59]. In people with type 2 diabetes, mitochondria were found to increase both in size and density after a 4-month lifestyle intervention of daily moderate–intensity exercise with moderate weight loss [45]. Increased fatty acid oxidation during endurance exercise permits sustained physical activity and delays the onset of glycogen depletion and hypoglycaemia.

Although not the liver itself, there is an important reduction in visceral adipose tissue with exercise. Visceral fat has been directly linked with liver inflammation and fibrosis, independent of IR and hepatic steatosis [60]. The precise mechanism of how visceral fat applies its detrimental effects on liver metabolism, fibrotic and inflammatory consequences remains unclear although influx of fatty acids and synthesis of cytokines and adipokines have been shown to promote liver fat

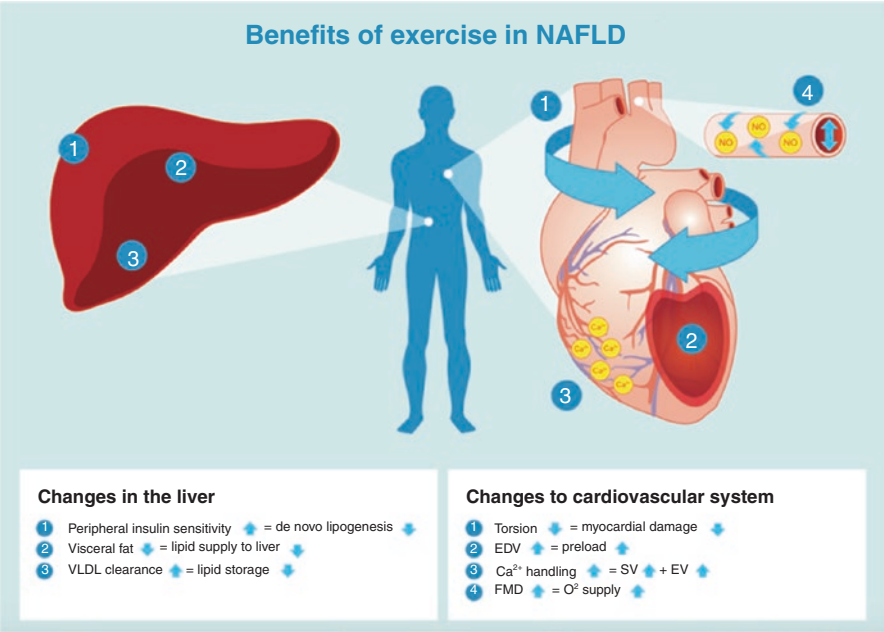

Fig. 16.2 Benefits of exercise and physical activity in NAFLD: changes in the liver and changes to cardiovascular system

accumulation, IR and inflammation [60]. There is much that is not known in the field of exercise and NAFLD, including the effect of exercise on inflammation (a key mediator in the progression of NAFLD), effect on gut microbiota and appetite for a start. However, given that people with NAFLD are at nearly double the risk of developing cardiovascular disease than those without [61], the beneficial effects of exercise on cardiovascular function [62] should be explored further. Indeed, it is possible that the major benefits for exercise in NAFLD are not in the liver, but in improving cardiovascular function. A schematic representation of the mediators of response to exercise in NAFLD can be seen in Fig. 16.2.

16.8 Aerobic Exercise and NAFLD

Aerobic exercise, sometimes referred to as cardio or cardiovascular exercise, is any activity that uses large muscle groups and can be maintained continuously over a period of time. It is generally rhythmic in nature and is a type of exercise that overloads the heart and lungs and causes them to work harder than at rest [63]. Multiple studies have highlighted the benefits of aerobic exercise, in NAFLD independent of weight loss [46–48]. The protocols used in these studies largely follow the guidelines for physical activity prescription in the general population of 150 mins moderate-to-vigorous intensity exercise per week [37, 38] and utilise

a combination of static cycling, walking/jogging and circuit-based exercise. For a large proportion of patients with NAFLD, these exercise levels may be too high a target to be aiming for initially as their baseline levels are significantly lower than this [10]. This is not surprising as figures from the Health Survey for England show that only 67% of men and 55% of women in the general population meet theses exercise targets. One barrier to exercise people often site is lack of time. High-intensity intermittent training (HIIT) is a relatively new method of exercising. HIIT consists of exercise divided into high-intensity bouts interspersed with recovery periods and can provide comparable or greater benefits to cardiorespiratory fitness than continuous moderate–intensity exercise of longer duration [64]. Studies have found that some volunteers prefer HIIT to continuous exercise routines as it is less time consuming [65, 66]. HIIT has also been shown to improve liver fat and cardiac function in patients with NAFLD [49] and is another option to offer patients in the clinical setting. It is worth noting that more vigorous aerobic exercise does not hold additional benefit for liver fat compared with moderate aerobic exercise [50, 51]—the majority of patients with NAFLD would benefit from a combined exercise approach, which targets not only liver health but also type 2 diabetes and CVD risk. Ultimately, exercise prescription for our patients with NAFLD should be individualised to promote adoption and long-term adherence to the exercise regimen and should take into consideration patients' other comorbidities, their baseline capabilities and personal preferences [67].

16.9 Resistance Exercise and NAFLD

Resistance exercise, often known as strength or weight training, works the muscles against a load. Resistance exercise provides an alternative to aerobic exercise; it improves muscular strength, muscle mass and metabolic control, safely and effectively, in vulnerable populations independent of weight loss [68]. It places less of a demand on the cardiorespiratory system and may therefore be accessible to more patients [69] thus proving a particularly useful tool in the management of our NAFLD patients with multiple comorbidities.

Evidence that resistance exercise can improve body composition is increasing, and it is now recommended by the American College of Sports Medicine and the American Heart Association as an integral component to any exercise programme [70, 71]. A meta-analysis comparing aerobic training with weight training concluded that weight training resulted in greater increases in fat-free mass [72]. An increase in muscle mass may improve insulin sensitivity by increasing the available glucose storage area, thereby reducing the amount of insulin required to maintain a normal glucose tolerance. An increased muscle mass may also improve fat oxidation due to an increase in the number of mitochondria.

Resistance exercise has been shown to decrease respiratory exchange ratio (RER) after exercise, indicating elevated fat oxidation [70]. This reduction in RER has been reported to last hours after a single bout of resistance exercise [71, 73]. This represents a shift towards greater fat relative to carbohydrate oxidation during

the post-exercise period. Enhanced fat oxidation, observed as an acute response to resistance exercise, is due to glucose sparing for the purpose of glycogen replenishment, thus resulting in fatty acids being the primary substrate for energy provision after resistance exercise.

Strenuous resistance exercise could be beneficial in weight control, not only because of the direct caloric cost of the activity and the residual elevation of the post-exercise VO_2 but also because of the greater post-exercise fat oxidation. Energy expenditure has been found to be elevated for as long as 38 h after an acute bout of heavy resistance exercise [74]. Results suggest that the energy required to recover from resistance training may be of significant use to a weight control/loss programme. For the first 24-h period following exercise, metabolism was increased by 21% and over a further 24 h by 19%. These differences could equate to 404 kcal and 369 kcal increases per day, respectively, for average build individuals [74].

- **Recommendations for exercise prescription in NAFLD** [67]
- **Aerobic (e.g. jogging, cycling):**
  - **150–300 min/week of moderate-to-vigorous intensity** (50–70% VO_2peak) **≥3 days/week**
- **Resistance (strength training):**
  - **2–3 sets of 8–12 repetitions** (70–85% 1RM) **2–3 days/week**
- For weight maintenance: ↑ volume of exercise
- For improvement in cardiorespiratory fitness and glycaemic control: ↑ intensity of exercise

16.10 Diet, Sedentary Behaviour, Physical Activity and Exercise

Although exercise has a significant and clinically meaningful effect on liver lipid (20–30% relative reduction), its effects are modest in comparison to weight reduction which can produce >80% reduction in liver fat [46]. This is important as, clinically, supporting people to manage their weight through diet approaches will produce greater changes in liver fat than exercise alone. However, completely disassociating exercise and diet may not be beneficial as data suggests that cardiorespiratory fitness is a determinant of response to dietary intervention in NAFLD, with those with a greater cardiorespiratory fitness having a greater response to dietary intervention [75]. This creates a difficult paradox where those with the lowest cardiorespiratory fitness, who will find exercise most difficult, also have the lowest response to diet-induced lifestyle interventions. Additionally, high levels of physical activity (i.e. 200–300 min/week) are crucial for weight loss maintenance [76],

and since physical activity has an independent effect in NAFLD treatment, it provides another treatment option for those who have difficulties in weight loss.

16.11 Physical Activity Measurement

In order to utilise physical activity/exercise as a treatment strategy in the management of NAFLD, we need a means to accurately measure levels of sedentary behaviour, physical activity and exercise. Sensitive and specific tools are required to best characterise the habitual patterns of activity in our patients and to monitor the effectiveness of lifestyle interventions. These tools may also assist clinicians in providing accurate feedback to the patient as to their current activity levels, and enable individual activity targets to be set, monitored and worked towards as part of the patient's treatment package. Several different methodologies exist for the measurement and assessment of physical activity and energy expenditure (EE). These methodologies range from expensive and objective laboratory measures such as doubly labelled water to subjective measures such as self-reported physical activity questionnaires. All of these tools have benefits and limitations, and their appropriate use depends on multiple factors, especially the context in which they are being used. The most clinically useful measures are discussed below:

Physical activity questionnaires: There are a large number of self-recall physical activity questionnaires. The most frequently used are the Baecke and IPAQ. Self-reported physical activity is valid [77–79] and useful in understanding broad differences in physical activity in large cross-sectional studies. However, these techniques are not sensitive to monitor changes in activity patterns or allow accurate determination of energy expenditure (EE) and are subject to recall error [80]. They can be useful to use on an individual patient basis to gain an estimate of current activity levels thus allowing the clinician to open the conversation about changing activity habits, but are not sensitive enough to detect small changes made through lifestyle interventions.

Heart rate monitors: Heart rate monitors are routinely used to measure physical activity in both research and recreation, with an increase in heart rate used as a surrogate marker for an increase in physical exertion. However, heart rate monitors are only accurate in measuring moderate–vigorous activities, as in lower intensity activities, confounding factors, such as stress, emotions, illness and caffeine intake, have a significant impact on results [81]. Heart rate monitors may therefore be deemed an inappropriate technique, when used in isolation, for measuring day-to-day activity which is generally of low–moderate intensity. They also do not provide information about the type of activity or activity patterns across the day/week.

Pedometers: Pedometers are simple devices, which use up and down motions as estimates of steps. Pedometers provide a low-cost means of crudely measuring physical activity. The major drawback to this method is that pedometers measure footfalls, and thus any activity undertaken which does not involve ambulation (e.g. weight lifting, biking, swimming) is inaccurately recorded. Pedometers also fail to

capture intensity, frequency or duration of activity. In most cases, pedometers prove accurate in counting steps; however, they are much less accurate in predicting EE, with error rates of ±30% [82].

Accelerometry: An accelerometer is an electromechanical device that will measure acceleration forces. Basic, uniaxial accelerometers measure acceleration of the body or body parts in one plane and take into account the speed, direction and duration of movements and convert these to movement counts to allow for estimation of EE. Biaxial or triaxial accelerometers provide information about movement in multiple directions, and show a better relationship to physical activity EE than uniaxial units [83]. All accelerometers are subject to motion artefacts, and cannot distinguish movement from activities such as driving a car, from actual "physical" activity. Error rate for accelerometry ranges from 14 to 30% against laboratory measures [84, 85] with uniaxial units prone to the greatest recording error due to their relative insensitivity to whole-body movement.

Multi-sensor array: Multi-sensor systems, or multi-sensor arrays, combine measures such as heart rate, accelerometry and body temperature to provide an overall more accurate picture of physical activity patterns. Multi-sensor arrays utilise pattern detection algorithms (typically determined by the respective manufacturer) to combine physiological signals detected from the different sensors to first identify the wearer's context, and then apply an appropriate formula to estimate EE from the sensor values [86]. These monitors are generally easy and comfortable to use and have an average error rate of 8–10% when compared to laboratory measures [86, 87].

16.12 Summary

In the absence of approved pharmacotherapies for NAFLD, lifestyle change remains the cornerstone of clinical care [88]. Structured exercise produces significant, but modest, improvements in liver lipid [46]. Evidence-based guidelines for sedentary behaviour and physical activity are lacking in NAFLD. General guidelines for physical activity of 150 min of moderate exercise per week or 10,000 steps per day are good rules of thumb, based on guidelines for the primary prevention of cardiovascular disease [39]. However, the current literature cannot inform us how much sitting is too much, we just know that it is better to sit less than to sit more. Furthermore, it is better to have more breaks in sedentary behaviour than less [89]. Targeting a reversal of sedentary behaviour may also provide an additional therapeutic avenue to complement physical activity and exercise as therapies for NAFLD, but has not been tested yet. There remains a significant lack of large-scale studies exploring physical activity and exercise in NAFLD, with and without dietary change/pharmacotherapy, limiting the generation of guidelines specific for NAFLD. Despite the relative infancy of evidence, the available data suggests that physical activity and exercise provide useful tehraputic tools for the prevention and management of NAFLD and NASH and should be supported.

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Pharmacological Options for NASH

17

Christiane Stern and Vlad Ratziu

Nonalcoholic steatohepatitis (NASH) is becoming the leading cause of chronic liver disease and a major health issue owing to its close association with the worldwide epidemics of obesity and diabetes [1]. A significant proportion of patients can experience disease progression with the occurrence of cirrhosis, hepatocellular carcinoma and end-stage liver disease [2]. This results in an increase in the overall and liver-related mortality [3, 4]. Patients at risk of disease progression need to be identified as not all individuals with metabolic risk factors will experience disease progression [5]. Prognostic markers have mostly been derived from histological studies and found that the degree of inflammation is the strongest and independent predictor for fibrosis progression [6]. Thus, therapies that could reduce liver inflammation would be the most meaningful option to control this disease.

While simple to recommend, diet and lifestyle measures as a first-line therapy for nonalcoholic steatohepatitis (NASH) are hardly a model of successful therapy as most clinicians can testify. They can be complex to implement, hard to sustain, and of limited efficacy in advanced stages of the disease. The need for specific pharmacotherapy is now acknowledged by practitioners, the pharmaceutical industry, and regulators and is largely expected by patients. The result is a clear move away from products developed second-hand for NASH (such as pioglitazone or metformin) or from generic, non-specific hepatoprotectors (such as pentoxifillin, ursodeoxycholic acid, or antioxidants) toward molecules developed and tested specifically for NASH that aim to correct one or several of the pathways of liver injury in this disease. The two most advanced molecules, obeticholic acid (OCA) and elafibranor, have shown encouraging data on improving hepatic histology. Both compounds appear to clear NASH, with OCA improving liver fibrosis and elafibranor improving the glycemic and lipid profile. Cenicriviroc is also being tested as an antifibrotic drug in NASH.

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17.1 What Are the Relevant Pharmacological Targets?

Our current understanding of the pathophysiology of NASH is that excessive fat accumulation coexisting with overweight, particularly when localized to visceral adipose tissue, promotes insulin resistance. Uninhibited lipolysis, a consequence of insulin resistance increases delivery of free fatty acids to the liver [7]. In addition, hyperinsulinemia and the subsequent increase in serum glucose will enhance a maladaptive hepatic lipogenic response and inhibit lipid disposal through beta-oxidation [8]. The resulting increase of intrahepatic flux of numerous lipid species promotes liver damage through multiple lipocytotoxic pathways: oxidative stress, mitochondrial dysfunction, apoptosis, free cholesterol toxicity, and endoplasmic reticulum stress [9]. The resulting cell injury and accompanying inflammation (part of which is modulated by cross-talk with the inflamed adipose tissue) sets the stage, in the long run, for liver fibrosis to occur.

This brief description suggests that the relevant mechanisms of action for NASH drugs could be: (1) weight loss agents; (2) insulin sensitizers; (3) antidiabetic drugs with antihyperglycemic properties; (4) hepatoprotectants with broad anti-inflammatory properties; and (5) antifibrotic drugs. These drugs can therefore be classified into two broad categories: drugs that improve the underlying metabolic conditions that promoted the emergence of NASH; and hepatoprotectants that specifically target the mechanisms of hepatic cell injury. As some pathways can be involved in both hepatic inflammation and insulin resistance, some drugs might belong to both categories. Alternatively, combination therapy with molecules that act on distinct metabolic and hepatoprotective pathways could also be envisioned. Depending on how vast the NASH drug pipeline will be, tailored therapy for particular patients could thus become a reality in the near future.

17.2 Where Do We Stand with Pharmacological Therapies?

An ideal drug candidate for NASH should reduce hepatic inflammation and liver cell injury, should correct the underlying insulin resistance, and should have antifibrotic effects. However, primarily "anti-NASH" drugs that have no direct antifibrotic effect could, theoretically, result in a subsequent reduction of fibrosis if a sustained resolution of NASH is achieved. Conversely, purely antifibrotic drugs with no anti-NASH activity and no interference with insulin resistance will leave the triggers for fibrogenesis intact. Therefore, even if an antifibrotic is effective, efforts to curb the underlying pro-fibrotic condition must be considered [10]. We will here review some of the novel anti-NASH agents that are now in late stages of drug development. Many other agents are in preclinical phases of development or in early human studies and will not be reviewed here. These agents that target fibrotic pathways, hepatic lipogenesis, endothelial adhesion molecules, apoptosis, miRNA, endotoxin, nuclear receptors among others are part of a very diverse and rich pipeline for NASH.

17.3 Available Agents with Limited Testing

17.3.1 Insulin-Sensitizing and Antidiabetic Agents

Metformin. Metformin is a safe and inexpensive compound that acts as an insulin-sensitizing agent by reducing hepatic glucose production and increasing peripheral insulin utilization. It reduces body weight. The efficacy in NASH is not proven. An open-label study showed histological improvement (reduction in a histological index) [11], but this was not confirmed in other open-label [12, 13] or randomized trials [14] and a meta-analysis [15]. It is possible that higher weight loss in some patients could explain histological improvement. The anti-steatogenic effect of metformin is weak and is consistent with its inability to restore serum adiponectin levels [16]. Metformin is not recommended for the treatment of NASH. Recent studies however have suggested an association between metformin use and reduced risk of hepatocellular carcinoma [17]. Other studies, in diabetic patients with NASH cirrhosis, have shown that continued treatment with metformin is associated with less episodes of cirrhosis decompensation [18]. Similarly, in a monocentric cohort of diabetics with NASH and advanced fibrosis or cirrhosis, metformin was associated with increased transplant-free survival and reduced risk of HCC [19].

Thiazolidinediones (glitazones) are PPAR gamma agonists, which are potent insulin-sensitizing agents and marketed for treatment of type 2 diabetes. They promote adipocyte differentiation into small, insulin sensitive adipocytes. With long-term treatment, fat storage is redirected from illegitimate storage sites, such as the liver and muscle, toward the adipose tissue, which alleviates hepatic and muscle insulin resistance and reduces lipotoxicity. Glitazones also increase adiponectin levels, an anti-steatogenic and anti-inflammatory cytokine, which is reduced in NAFLD. Glitazones are the best studied pharmacological class in NASH. Several open-label and controlled studies are available with pioglitazone or rosiglitazone, as well as one pediatric trial with pioglitazone. Unfortunately these trials are heterogeneous for daily doses, duration of therapy, and included population (diabetics or non-diabetics) [20]. The largest trial so far is a NASH CRN-sponsored trial comparing pioglitazone at a low dose of 30 mg/day vs. vitamin E (400 IU/day) vs. placebo for 2 years in patients without full-blown diabetes [21]. Although the primary endpoint (histological improvement, defined as a reduction in the NAS without worsening of fibrosis) was not formally met, pioglitazone improved all individual histological features (except for fibrosis) and in particular achieved clearance of steatohepatitis—currently considered the optimal end point in NASH trials [22, 23]. Importantly, when the analysis was limited to patients with well-defined steatohepatitis upon inclusion, pioglitazone reached the primary endpoint with an even more stringent than usual p-value of 0.025. Thus the PIVENS trial [21] should not be seen as a negative trial for pioglitazone but rather an underpowered trial displaying a strong trend toward histological improvement for this drug. The histological benefit occurred together with ALT improvement and partial correction of insulin resistance [21]. In particular, a short-term 6-month treatment with pioglitazone

improves adipose tissue insulin sensitivity, which correlates with hepatic histological improvement [24]. Similar results were reported in two other randomized trials of 6 months and 1 year duration [25, 26]. For reasons still unclear, rosiglitazone failed to show histological benefit in the hallmark histological lesions of steatohepatitis, even though there was a significant reduction in steatosis and a biochemical (ALT) and metabolic (HOMA) response [27]. Most of these effects were obtained in the first year of therapy, and prolonged therapy for up to 3 years did not result in further improvement [28].

The enthusiasm for glitazones as a treatment for NASH is seriously dampened by the side effects of these drugs [20]. First and most immediate is weight gain, which is due to adipose tissue buildup and is not always reversible upon discontinuation. Bone fractures in women have been reported with both glitazones and seem to be due to an increased rate of bone loss. Congestive heart failure is a rare complication, yet it warranted a black box warning for both glitazones. Recently, the demonstration of an increased risk of bladder cancer with pioglitazone justified its market withdrawal in some European countries. Finally, an increased risk of cardiovascular events, especially myocardial infarction with rosiglitazone, has been hotly debated and the magnitude of the risk is still uncertain [20]. Nonetheless, rosiglitazone has received a black box warning in the USA and has been withdrawn from the market in many European countries.

17.3.2 Antioxidants

Vitamin E (Vit E). Vit E (alpha tocopherol) is a naturally occurring antioxidant that inhibits TGF-beta, prevents hepatic stellate cell activation, and improves liver necrosis and fibrosis in animal models. The PIVENS study showed that a 2-year treatment with Vit E at 800 IU/day in adult patients significantly reverses steatohepatitis and improves all histological features of NASH (except fibrosis) compared to placebo [21]. Interestingly, this beneficial effect of Vit E was not associated with an improvement in insulin sensitivity. The TONIC trial confirmed the histological efficacy of Vit E 800 IU/day in a pediatric population: after 2 years of treatment Vit E cleared NASH and improved ballooning more often than placebo [29]. Of note, there was no effect on steatosis or inflammation, and, despite prolonged therapy, still no effect on fibrosis. Also, these histological endpoints were only secondary endpoints. The reduction in ALT, which was the primary endpoint, was not achieved by Vit E, as the trend was not statistically significant [29]. A smaller 2-year pediatric Italian trial did not show any histological or biochemical efficacy of the combination of VitE (600 mg/day) and Vit C vs. placebo [30]. In this trial, however, an intense diet and lifestyle intervention program was successfully implemented in both groups and resulted in similar weight loss and improvement in insulin resistance, thus blurring the differences between the antioxidant and control arm.

Pending confirmation by other investigators, the histological results of the two NASH CRN-sponsored trials on Vit E [21, 29] seem encouraging. Nonetheless the controversy around the long-term safety of Vit E supplements dictates restraint

in generalizing recommendations of use for Vit E: several meta-analyses suggest increased mortality in patients taking Vit E supplements [31, 32]; one meta-analysis showed a 20% increase in the risk of hemorrhagic stroke [33]; and another large trial suggested an increase in the risk of prostate cancer in men older than 50 years [34].

17.4 Agents in Development

17.4.1 FXR Agonists and Obeticholic Acid

Recent discoveries have identified bile acids as key regulators of liver and metabolic homeostasis. Their action is mediated through nuclear hormone receptors such as the farnesoid X receptor (FXR) and TGR5 [35]. FXR activation results primarily in a reduction of bile acid synthesis from cholesterol by altering expression of a host of genes but mainly by downregulating CYP7A1 [36]. This limits the size of the circulating bile acid pool and promotes choleresis thus protecting against the toxic accumulation of bile acids. Obeticholic acid (OCA), a first-in-class FXR agonist, is a synthetic bile acid with picomolar agonistic activity on FXR [36]. The bile acid effects have translated into clinical efficacy in patients with primary biliary cirrhosis [37] with a reduction in phosphatase alkaline, a biochemical surrogate for clinical events in the natural history of the disease [38]. Based on these results, it is expected that OCA will be approved for this indication. FXR activation also has a wide range of metabolic effects: inhibition of hepatic neoglucogenesis and hepatic glucose production, reduction of lipogenesis and enhancement of beta-oxidation, improvement in peripheral insulin sensitivity [39]. Interestingly, FXR activation has also anti-inflammatory actions [40] with resultant protection against liver inflammation and fibrosis in experimental models of NASH [41, 42].

A small randomized trial in type 2 diabetic patients with NAFLD showed an improvement in hepatic and muscle insulin sensitivity as measured by the euglycemic clamp, a modest but dose-related weight loss, and a reduction in ALT levels [43]. This study provided the proof of principle of an improvement of insulin sensitivity and possibly NAFLD in humans. It was followed by a much larger trial that tested the oral administration of 25 mg OCA QD vs. placebo over 72 weeks of therapy in non-cirrhotic NASH patients [44]. The therapeutic phase of the FLINT trial was stopped early, partly because a preplanned interim analysis showed improved histology in more patients on OCA than on placebo (45% vs. 21%). The primary endpoint was a two-point *reduction in the composite Nonalcoholic Fatty Liver Disease Score (NAS)* without worsening of fibrosis. However, beyond this composite end point, OCA was able to significantly improve all histological lesions constitutive of NASH including liver fibrosis. Although the trial was not designed for fibrotic endpoints, there was a significant reduction in the fibrosis score (one stage) in 35% of OCA-treated patients vs. 19% in the placebo arm. The reduction in fibrosis was observed regardless of the baseline fibrosis stage. The study included patients at high risk of progression (half of the participants had type 2 diabetes) and "non-responders" to vitamin E (20%). The primary endpoint was reached in secondary

analyses of all subgroups of patients. There was a trend in favor of a higher rate of resolution of NASH in the OCA group (22% vs. 13% in the placebo group) which became significant (19% vs. 8%, $p < 0.05$) in a subgroup analysis restricted to patients with well-characterized NASH at baseline. As far as safety and tolerability, two issues emerged: pruritus and an increase in LDL cholesterol. Pruritus occurred in 23% of OCA-treated patients vs. 6% in the placebo group, but discontinuation was very rare (only one patient). It is however a concern as the NASH population is overwhelmingly asymptomatic. Further studies will test whether lower doses of OCA reduce the incidence of pruritus. An increase in LDL cholesterol occurred early on therapy, plateaued with continued therapy then reversed once the drug was discontinued. Post hoc analyses showed that statins, when initiated during the trial, were able to mitigate the excursion in LDL. Future studies are needed to better characterize alterations in lipid profile and to determine if this results in an increase in cardiovascular risk, if any. Interestingly, in animal models of atherosclerosis, FXR agonists reduce atherosclerosis and vascular cholesterol load and inflammation. A large phase 3 trial [REGENERATE trial (NCT02548351)] comparing three groups (OCA 10 mg QD vs. OCA 25 mg QD vs. placebo) is ongoing in non-cirrhotic NASH patients. The 18-month preliminary analysis [45] of 931 patients (ITT population) confirmed that 25 mg OCA QD induces a fibrosis reduction of at least 1 stage with no worsening of NASH (23.1% vs. 11.9%, $p = 0.0002$). Pruritus was confirmed as the main adverse event in up to 51% in OCA 25 mg QD group, with the highest incidence in the first 3 months and leading to 9% of discontinuation as per protocol requirement. Currently a large 2-year study in cirrhotic patients is ongoing (REVERSE trial). There is rationale for a benefit of OCA in cirrhosis: in rodents, OCA reduces bacterial translocation by increasing the expression of intestinal tight junction proteins which resulted in a normalization of the endotoxin-TLR4 signaling [46, 47]. Also, OCA can reduce the intrahepatic vascular resistance and improving endothelial vasorelaxation by restoring hepatic e-NOS activity [48]. This suggests beneficial effects on portal hypertension which together with reduced risk of infections due to reduced bacterial translocation could result into clinical benefit in cirrhotic patients.

The main side effects observed with OCA has led to the development of second generation, non-bile acid FXR agonists with the hope of a reduced incidence of pruritus and lipid changes. Early trials are ongoing with several compounds such as tropifexor, cilofexor, and others. Data on efficacy are not yet available.

17.4.2 PPAR Alpha/Delta Agonists and Elafibranor

Another innovative insulin sensitizer is elafibranor, a dual PPARα/δ agonist. *PPARs* (α, β, and γ) are fatty acid-activated nuclear receptors that have a wide range of physiological actions. PPARδ activation emerged as a potent metabolic regulator that induces hepatic fatty acid β-oxidation, inhibits hepatic lipogenesis [49], reduces hepatic glucose production, and improves hepatic inflammation [50, 51]. PPARα is a major regulator of fatty acid disposal through mitochondrial beta-oxidation, but has also anti-inflammatory actions as it inhibits inflammatory genes induced

by NF-kB and acute phase response genes induced by IL6 [52]. Combining these two modes of action can thus improve many of the pathways of injury involved in NASH. Animal data confirmed that elafibranor has hepatoprotective effects in dietary models of NASH or fibrosis with a reduction in steatosis, hepatic inflammation, and pro-inflammatory genes [53]. Importantly, this compound exhibited antifibrotic properties in fibrosis models that were independent of metabolic and insulin resistance abnormalities [53], thereby suggesting a universal antifibrotic potency in rodents. Elafibranor is a PPAR modulator with preferential activity on PPARα and additional activity on PPARδ, but no PPARγ actions [54]. It undergoes extensive enterohepatic cycling and is liver-targeted with little or no muscle action [55]. Human studies performed in abdominally obese, insulin-resistant patients, with or without diabetes, have shown that elafibranor improves hepatic and peripheral insulin sensitivity, dyslipidemia, inflammatory markers, and liver function tests [54, 56].

The results of a large, international, phase IIb trial, the GOLDEN505 trial have been reported [57]. In this randomized trial, 274 NASH patients received elafibranor 80 mg/day, 120 mg/day, or placebo for 1 year. While the lower 80 mg dose did not improve histology, the higher dose was more effective than placebo at inducing NASH resolution without fibrosis worsening. The optimal definition for this histological outcome is still under debate, but these positive results were obtained with a modified, more stringent definition that is consensually emerging. There was no effect on fibrosis (1-year trial duration only) although patients who cleared steatohepatitis (responders) had an improvement in fibrosis after 1 year of therapy, while nonresponders did not. Importantly, improvement in the activity score (i.e. reduction in hepatocyte ballooning and inflammation) was correlated with reduction in fibrosis [58]. This validates the concept that resolution of NASH will be followed by a reversal of fibrosis, a cornerstone of the current surrogate endpoints used in drug development. As anticipated from earlier phase 2 trials, elafibranor improved lipid parameters, glucose homeostasis and insulin sensitivity as well as systemic inflammatory markers. Remarkably, the cardio-metabolic improvement was achieved on top of standard of practice management of the comorbidities in these patients with metabolic syndrome. The drug was well tolerated although a few patients had an increase in creatinine, which was reversible after discontinuation of treatment. The increase was less than that observed with fibrates and, similarly to fibrates, it is not expected to be associated with renal insufficiency. A phase 3 trial, RESOLVE-IT, evaluating the efficacy and safety of elafibranor 120 mg once daily for 72 weeks, has been stopped because the trial did not meet the predefined primary endpoint of NASH resolution without worsening of fibrosis in the ITT population of 1,070 patients at interim analysis. The response rate in the 717 patients enrolled on study drug was 19.2% for patients who received elafibranor 120 mg compared to 14.7% for patients in the placebo arm.

17.4.3 Chemokines and Cenicriviroc

Chemokines are chemotactic cytokines specialized in leukocyte recruitment at sites of tissue injury, inflammation, and fibrosis. Chemokines and their receptors form a complex network of redundant ligand-complex binding as one receptor may bind

different chemokines, but their overall effect is the promotion of local inflammatory and fibrotic response [59]. CCL2 (a.k.a. *monocyte chemoattractant protein*, MCP1) and CCL5 (RANTES) are particularly involved in liver and adipose tissue inflammation and hepatic fibrosis [60–62]. Cenicriviroc (CVC) is a selective inhibitor of CCR2 and CCR5 with nanomolar potency. It was developed initially as an anti-HIV agent as CVC blocks the use of CCR5 as a co-receptor for entry into host cells by HIV. CVC blocks the binding of MCP-1 to CCR2 and of RANTES, *macrophage inflammatory protein-1α* (MIP-1α) and MIP-β to CCR5. There is a strong rationale for the use of CVC in NASH. CVC decreases recruitment, migration, and infiltration of pro-inflammatory monocytes to the site of liver injury mainly via CCR2 antagonism, thereby having the potential to reduce chronic liver inflammation. CVC also disrupts co-receptor and cytokine signaling pathways or "cross-talk" of intrahepatic immune cells within the inflamed liver via CCR2 and CCR5 antagonism, resulting in decreased Kupffer cell and hepatic stellate cell activation and migration, and therefore reduced fibrogenesis. CVC demonstrated significant antifibrotic effects in diet-induced (mouse model of NASH with streptozotocin and high-fat diet [63]) and chemically induced (rat thioacetamide [TAA] [64]) models of liver fibrosis, as well as in a model of kidney fibrosis. It also reduced lobular inflammation and hepatocyte ballooning in the dietary NASH model. Studies in up to 48 weeks in HIV-infected individuals did not show any safety concern.

A large, randomized phase 2b trial in NASH, the CENTAUR trial has been conducted in patients with fibrotic NASH or NASH at high risk of progression [65]. This trial tests CVC vs. placebo over a 2-year period. The primary analysis was performed after 1 year of therapy [66]. Entering year 2, half of the patients in the placebo arm were switched to the active arm and exploratory results at year 2 were reported. At neither time points, cenicriviroc improved the activity score or resolved NASH. However, there was a doubling of the rate of a one stage or more fibrosis regression at year 1 vs. placebo. During the second year of therapy, there was no additional antifibrotic effect, but exploratory analyses on a small number of patients suggest a higher durability of the antifibrotic effect. A registrational, phase 3 trial of Cenicriviroc is ongoing (the AURORA study).

17.4.4 Fatty Acid-Bile Acid Conjugates and Aramchol

Aramchol is a first-in-class, novel synthetic small molecule produced by conjugating two natural components, a fatty acid, arachidic acid, and a bile acid, cholic acid linked by a stable amide bond. It was initially synthesized to treat gallstones as the saturated fatty acid has cholesterol-solubilizing properties and the bile acid enabled secretion into the bile and entry into the enterohepatic circulation [67]. However, empirical observations of animals fed a high-fat, lithogenic diet documented a strong reduction in liver fat that occurred much earlier than did gallstone dissolution [68]. The anti-steatogenic mechanism is probably related to the inhibition of *stearoyl CoA desaturase1* (SCD1) activity well documented in human liver [69]. This results in decreased synthesis of mono-unsaturated fatty acids and of

triglyceride stores. Moreover, aramchol activates cholesterol efflux by stimulating the ABCA1 transporter, a universal cholesterol efflux pump [70] which can explain the anti-atherogenic effects in some animal models [69]. Since liver-specific SCD1 inhibition in rodents reversed hepatic insulin resistance and reduced neoglucogenesis [71] several SCD1 inhibitors were tested as a treatment of diet-induced metabolic complications. However, systemic inhibition of SCD1 resulted in severe skin and eye side effects, and most of them have been discontinued [72]. Aramchol does not induce these side effects possibly because of the liver targeting or the partial and not complete inhibition of SCD1. A small phase 2a study performed in patients with biopsy documented NAFLD tested two doses of aramchol vs. placebo over a 3-month period and did not raise any significant safety concern [73]. The higher, 300 mg daily, dose resulted in significant reduction in liver fat as measured by magnetic resonance spectroscopy (MRS). There was also a trend toward an increase in serum adiponectin and an improvement in flow-mediated dilation [73], an early marker of endothelial dysfunction in patients with NASH [74].

A large international phase 2b trial is ongoing in patients with histologically documented NASH, high liver fat content measured by MRS and several features of the metabolic syndrome (NCT 02279524). This trial of 1-year duration tests still higher doses of daily aramchol, 400 and 600 mg. The results, reported in abstract form, have shown a reduction in liver fat by magnetic resonance spectroscopy and also histologically a higher rate of NASH resolution in the high-dose aramchol arm than in placebo with a numerically higher rate of fibrosis regression which was not statistically significant. A registrational phase 3 trial is planned for testing the histological efficacy on a much larger scale.

17.4.5 Acetyl-Coenzyme A Carboxylase Inhibitors

Hepatic fat is mainly derived from free fatty acids that are released from the adipose tissue while de novo lipogenesis (DNL), the formation of new fatty acids from excess carbohydrates and amino acids, contributes for only about 5% of liver fat content [75]. However, in the setting of NAFLD, DNL is increased 2–3 times. The cytosolic enzyme acetyl-CoA carboxylase (ACC1) converts acetyl-coenzyme A (CoA) to malonyl-CoA which is a key substrate for fatty acid synthesis. This is the rate-limiting step in DNL, and it is 2–3 times greater in the setting of NASH. A second isoform of ACC (ACC2) is located in the mitochondria and is known to inhibit carnitine palmitoyltransferase I, the carrier protein of fatty acids into mitochondria for ß-oxidation, resulting in the oxidation of free fatty acids. Therefore, ACC inhibition both limits the production of fatty acids and promotes their breakdown.

Firsocostat (GS-0976) is a liver-targeted, inhibitor of ACC1 and ACC2 in development for the treatment of NASH expected to decrease DNL and increase mitochondrial β-oxidation. In preclinical and animal models, ACC blockade decreased hepatic steatosis, inflammation, and insulin resistance [76]. In a phase 2 trial of 126 non-cirrhotic NASH patients treated for 12 weeks [77] firsocostat reduced liver fat more than placebo: 48% of NAFLD patients receiving 20 mg of GS-0976 had

a relative reduction of ≥30% from baseline in MRI-PDFF versus 15% of patients receiving placebo. A notable side effect was hypertriglyceridemia (>500 mg/dL) observed in 14–18% of patients on active drug, predominantly in those with pre-existing triglyceride levels higher than 250 mg/dL; it was asymptomatic and reversible upon treatment with fibrates. Based on these encouraging results trials testing combined therapy of firsocostat with selonsertib and non-biliary FXR agonists are currently underway.

17.4.6 Incretin Mimetics and Liraglutide

Among existing therapies for type 2 diabetes, incretin mimetics which are glucagon-like peptide-1 receptor (GLP-1R) agonists hold promise for the treatment of NASH. GLP-1, a peptide product of the L cells of the small intestine and proximal colon, stimulates insulin secretion from the β cells and inhibits glucagon secretion from the α cells in a glucose-dependent manner [78]. GLP-1 also enhances satiety and delays gastric emptying [78]. However, because of their short half-life due to rapid degradation by specific enzymes (such as dipeptidyl peptidase, DPP-IV), native GLP-1 cannot be used as a pharmacological agent. GLP-1R agonists have a much longer half-life than natural GLP-1 allowing either a daily or a once-weekly administration [79]. There seems to be some controversy over the presence of receptors for GLP-1 in hepatocytes and stellate cells. Some studies have shown the presence of a cognate receptor for GLP-1 on human hepatocytes [80]; signaling through these receptors improves hepatic insulin sensitivity [81] by inducing phosphorylation of key signaling pathways [80]. GLP-1 R binding in hepatocytes results in an induction of PPARα and γ expression, which increases disposal of hepatocyte fatty acids by beta-oxidation and lipid export [81, 82]. In vivo studies have confirmed an anti-steatogenic effect of exendin in mice [81, 83]. Several potentially beneficial effects have been demonstrated in humans by metabolic studies including the euglycemic clamp: patients with NAFLD had decreased de novo lipogenesis, decreased adipose tissue lipolysis, and reduced hepatic glucose production upon administration of 1.8 mg liraglutide daily [84]. Moreover, because it induces weight loss, liraglutide at the dose of 3 mg/day [85] is now approved for treatment of obesity or overweight with comorbidities. Some of the effects of GLP1 R agonists seem to be mediated independent of weight loss: there are human data showing that an acute administration of exenatide improves hepatic and adipose tissue insulin resistance before any changes in weight occur [86]. Other GLP1-R agonists are approved for glycemic control in diabetic patients.

Data from large registration trials have shown that diabetic patients treated with liraglutide improved ALT levels and possibly steatosis, measured by CT-scan imaging [87]. Taken together all the above data form a compelling rationale for testing liraglutide in patients with NASH. A British study randomized 52 NASH patients and analyzed 23 of them treated with liraglutide, 1.8 mg/day, and 22 with placebo, in a randomized controlled trial of a 1-year duration [88]. Patients treated with liraglutide experienced more often reversal of NASH (39% vs. 9%, $p < 0.02$) and less

often progression of fibrosis. There was no significant effect on lobular inflammation and ALT and only a marginally significant effect on hepatocyte ballooning, an indication of the very small sample size of this trial. Hence, these results, although encouraging, especially in the light of the preclinical data and the weight loss effect, clearly need further confirmation before any recommendations can be made.

17.4.7 Antifibrotic Agents: Simtuzumab, Galectin-3 Inhibitor, and Caspase Inhibitors

Since the overall objective when treating NASH patients is to reduce the progression to cirrhosis, it would be important to have antifibrotic drugs directly blocking the fibrogenic process. There are very few well-conducted trials of antifibrotic agents and those that are available are negative [89–92]. Lysil oxidase and lysil oxidase-like (LOXL) are a family of enzymes expressed and secreted by fibrogenic cells and that catalyze oxidative deamination of lysyl and hydroxylysine residues in collagen precursors and elastin [93]. This results in covalent cross-linking of the extracellular matrix, a phenomenon that is believed to greatly contribute to the deposition and stabilization of the hepatic scar [94]. LOXL2, a member of the LOXL family, is upregulated in hepatocytes and its expression is correlated with collagen deposition in various hepatic fibrotic diseases [95] including steatohepatitis in humans [96]. LOXL2 regulates fibroblast activation, TGF-β signaling, and latent TGF-β activation [96]. Experimental studies have shown that inhibition of LOXL2 with an inhibitory monoclonal antibody results in a reduction in liver and lung fibrosis [96]. Simtuzumab is a humanized monoclonal IgG4 antibody with a long half-life of 10–20 days and that can be administered either IV or subcutaneously. Unfortunately two large trials in patients with NASH and bridging fibrosis and in cirrhotic NASH failed to demonstrate any antifibrotic potency on the histological stage of fibrosis or on the hepatic venous pressure gradient (HVPG) [97].

Galectins are a family of proteins that bind to galactose residues present on glycoproteins from extracellular matrix components (collagens, laminin, fibronectin, integrins, elastin) and also on cell surface proteins such as CD4, CD8, or TGF-beta receptors [98]. Galectin-3, a member of the galectin family expressed at high levels on macrophages, regulates multiple cellular processes including cell adhesion and migration, immune cell function, and inflammation [99]. It is upregulated in hepatic human fibrosis and promotes fibrosis in vitro and in vivo [100]. GR-MD-02, a complex polysaccharide polymer (a galactoarabino-rahmnogalacturonan) is a pharmacological inhibitor of galectin-3 that reduces liver fibrosis and portal hypertension in a thioacetamide model of fibrosis/cirrhosis [101]. The antifibrotic effects were confirmed in a dietary NASH model in diabetic mice where GR-MD-02 prevented accumulation of collagen and reduced stellate cell activation [102]. Remarkably the drug also improved hepatocyte ballooning and lobular inflammation and reduced fat accumulation; these anti-NASH effects are probably related to a reduction in iNOS, a marker of inflammation, and in CD-36 expressing pro-inflammatory macrophages [102]. A phase I dose-ranging study has shown good safety and tolerability in

humans receiving this compound intravenously (NCT01899859). A larger, phase 2a study in NASH patients with cirrhosis and portal hypertension testing intravenous infusions of GR-MD-02 every 2 weeks for 1 year failed to show an overall benefit on HVPG.

Caspases are a family of 11 intracellular cysteine proteases mediating apoptosis and regulating inflammatory and immune responses to dying cells. They produce hemodynamically active, pro-inflammatory microparticles that cause intrahepatic inflammation, vasoconstriction, and extrahepatic splanchnic vasodilation. Excessive hepatocyte apoptosis has been described in patients with NASH and considered a drug target as it induces inflammation and fibrosis. Emricasan (IDN-6556), an oral pan-caspase inhibitor, decreases hepatic apoptosis, inflammation, and fibrosis in animal models of acute hepatitis and chronic models of nonalcoholic steatohepatitis (NASH) [103, 104]. In a multicenter randomized study, 86 patients with cirrhosis Child-Pugh class A or B and MELD scores 11–18 received Emricasan 25 mg BID or placebo for 3 months [105]. Emricasan treatment improved MELD and Child-Pugh scores in patients with high MELD ($\geq$15) due to improvements in INR and total bilirubin. In another study including 23 compensated cirrhotics with portal hypertension (HVPG above 5 mmHg), Emricasan was administered for 28 days and induced a significant decrease in HVPG in patients with severe PH (HVPG $\geq$12 mmHg) at baseline [106]. Larger studies will be needed to better characterize the safety profile of Emricasan and the potential clinical benefit.

17.5 Conclusion

Drug development for NASH has accelerated strongly over the past few years. Earlier studies such as the PIVENS trial have provided the proof of principle that histological improvement and even NASH resolution is possible with drugs such as insulin sensitizers (glitazones) or antioxidants (vitamin E) [21]. Retrospective studies have documented the prognostic significance of histological lesions in NAFLD [107, 108], suggesting that these lesions could be acceptable surrogates of disease control on therapy. Tools for a precise histological description and classification have been refined from the NASH CRN classification [109] to the FLIP/SAF algorithm [110]. Major advances also occurred in the regulatory field. Both the European and the American drug agencies now agree that NASH is a valid indication for therapy and as such, it can follow a regulatory path for drug approval. Trial outcomes with clinical and regulatory value have been defined and are currently being used in several large trials of new drugs in NASH [10]. What remains to be done is the discovery and validation of biomarkers that would help diagnose patients at risk of advanced or progressive NASH and also monitor disease progression. Renewed and sustained efforts for drug discovery and dedication from physicians to recruit and complete clinical trials will be key to providing patients with NASH with safe and effective drugs in the near future.

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Bariatric Surgery and NASH: A Feasible Option

18

Lidia Castagneto-Gissey, James R. Casella-Mariolo, and Geltrude Mingrone

18.1 Background

In 1992, the National Institutes of Health (NIH) issued the eligibility criteria for bariatric surgery [1], which are currently endorsed by the majority of scientific societies [2, 3]. The NIH criteria include a body mass index (BMI) ≥ 40 kg/m^2 or a BMI between 35 and 40 kg/m^2 in individuals with high-risk comorbidities, such as decompensated type 2 diabetes or cardiovascular risk factors.

The joint statement of second Diabetes Surgery Summit (DSS-II), an international consensus conference, however, suggests that gastrointestinal "surgery should also be considered for patients with T2D and BMI 30.0–34.9 kg/m^2 [4] if hyperglycemia is inadequately controlled despite optimal treatment with either oral or injectable medications." Adjustments on BMI should also be made in relation to the ethnicity and body fat distribution.

Non-alcoholic steatohepatitis (NASH) can be plenty considered as a high-risk comorbidity of obesity.

Indeed, both non-alcoholic fatty liver disease (NAFLD) and NASH are regarded as the liver manifestation of the metabolic syndrome, which is a cluster of clinical and metabolic parameters including obesity, insulin resistance, hyperlipidemia, and hypertension [5].

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Obesity is the most common risk factor for NAFLD with more than 95% of the patients undergoing bariatric subjects being affected by NAFLD [6]. Instead, data on the prevalence of NASH in subject who underwent bariatric surgery are hectic, with some authors reporting a prevalence of 35% [7], others of 45% [8], and yet others of only 7% [9].

Regarding bariatric surgery to treat NAFLD, the EASL–EASD–EASO Clinical Practice Guidelines for the management of NAFLD [10] state with evidence B that "by improving obesity and diabetes, bariatric (metabolic) surgery reduces liver fat and is likely to reduce NASH progression; prospective data have shown an improvement in all histological lesions of NASH, including fibrosis."

The American Association for the Study of Liver Diseases in its recent practice guidelines [11] outline that "foregut bariatric surgery can be considered in otherwise eligible obese individuals with NAFLD or NASH," but that "it is premature to consider foregut bariatric surgery as an established option to specifically treat NASH."

18.2 Types of Bariatric Operations

18.2.1 Intragastric Balloon

Endoscopic bariatric therapies fill in the invasiveness and efficacy gaps in the spectrum of options currently available for the management of overweight and obesity. Intragastric balloons (IGBs) (Fig. 18.1) have been demonstrated to be effective therapeutic options for the treatment of obesity and obesity-related metabolic conditions, holding a low rate of adverse events [12, 13]. IGBs can be either resorbable

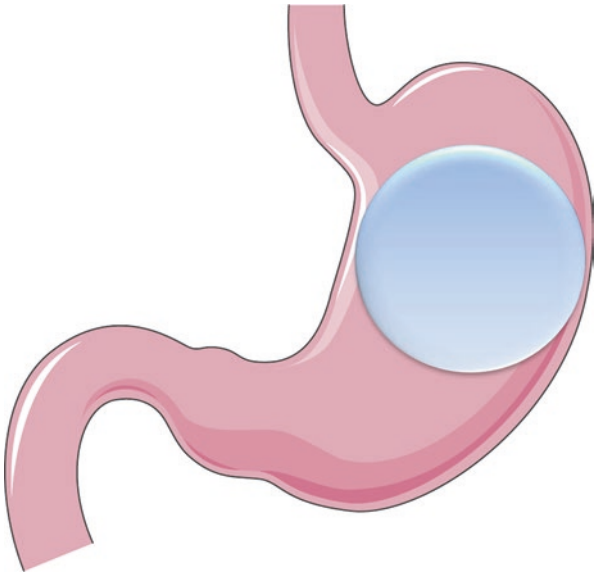

Fig. 18.1 Intragastric balloon occupying part of the stomach. The majority of the balloons are positioned via endoscopy while a new device can be swallowed as a small capsule connected to a tubing system and is then inflated with normal saline once it has reached the stomach

or non-resorbable. Non-resorbable IGB placement and removal requires sedation and upper endoscopy and mainly include the following: BioEnterics (BIB, Inamed Corporation, Arklow, County Wicklow, Ireland and Bioenterics Corporation, carpentry, California, USA) and Orbera (Apollo Endosurgery, Austin, TX, United States, now Allergan). On the other hand, Elipse Balloon System (Allurion Technologies, Natick, MA, USA) is a novel IGB device that requires neither endoscopy nor sedation for placement or removal. In fact, it can be swallowed as a small capsule connected to a tubing system and is then inflated with normal saline once it has reached the stomach. An abdominal X-ray is performed to confirm the correct balloon positioning. After about 16 weeks, the balloon is designed to spontaneously deflate and is eliminated through the gastrointestinal tract.

Early complications mainly comprise epigastric pain and nausea that develop in a majority of patients (70–90%) several hours after IGB insertion; such symptoms, however, usually regress 7 days from IGB placement. Early endoscopic IGB removal due to digestive intolerance is reported in 2.43%.

Late complications have been inconsistently described by various authors and include gastroesophageal reflux disease (GERD) esophagitis (1–11%), gastroduodenal ulcers (0.4%), gastric perforation (0.21%), hypokalemia (6–8%), and kidney failure (1–4%).

Deflation or rupture of the IGB is a potentially threatening complication, with rates ranging from 19 to 27% in earlier studies, to 0–4% in later ones. This can cause migration down to the ileo-cecal valve causing intestinal obstruction. Bowel obstruction may require surgical, endoscopic, or combined IGB removal and is described in 0.17% of cases [13, 14].

18.2.2 Adjustable Gastric Banding

Adjustable gastric banding (AGB) (Fig. 18.2) is a solely restrictive bariatric surgical procedure, involving an inflatable silicone band, placed around the proximal stomach, and connected to a tubing-port system placed subcutaneously. The gastric portion located just above the band forms a pouch with a capacity of approximately 10–20 mL. The AGB can be subsequently inflated through the tubing-port system. The small gastric pouch is quickly replenished with food and slows down the bolus passage, leading to early satiety.

In 2011, the Food and Drug Administration (FDA) revised its indications by expanding the approved BMI category to 30–40 kg/m^2 [15]. As a simple, rapid, and reversible operation, AGB gained widespread popularity between 2003 and 2008, followed by a steep decline after this period [16].

Long-term outcome studies have revealed a rather variably elevated rate of late complications (1.1–60%) and reoperations (0.92–60%). The major long-term complications after AGB include persistent nausea and vomiting, dysphagia, GERD, port-site infection, tubing system malfunction, and gastric pouch dilatation, with the most severe adverse events consisting of band slippage, erosion, and migration.

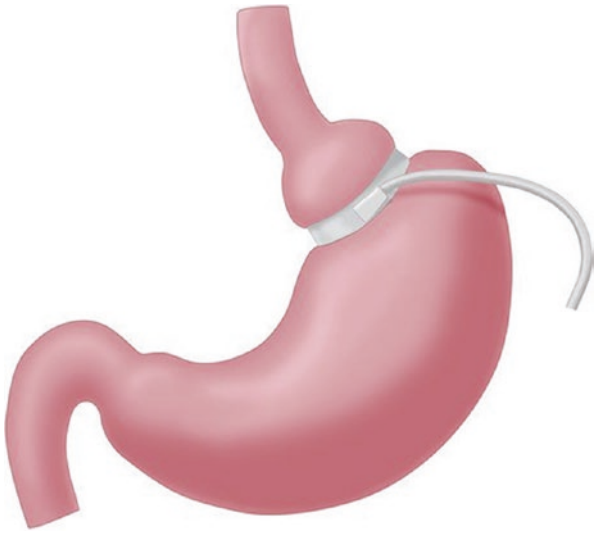

Fig. 18.2 Adjustable gastric banding (AGB) involves an inflatable silicone band, placed around the proximal stomach and connected to a tubing-port system placed subcutaneously. The gastric portion located just above the band forms a pouch with a capacity of approximately 10–20 mL. The AGB can be subsequently inflated through the tubing-port system to reduce the gastric volume thus inducing satiety

18.2.3 Sleeve Gastrectomy

Sleeve gastrectomy (SG) (Fig. 18.3) entails the longitudinal resection of the stomach along its greater curvature, carefully performing a complete excision of the gastric fundus, part of the body and antrum, yet maintaining a portion of the latter and the pylorus itself. The tube-shaped gastric sleeve maintains a capacity of approximately 60–150 mL.

SG was initially considered the first step of a more complex procedure (i.e., biliopancreatic diversion with duodenal switch). Nonetheless, with time this operation proved to generate superimposable weight loss and comorbidity resolution rates to that of other long-lived bariatric surgical procedures (namely RYGB) [17]. This allowed SG to become the most commonly performed bariatric operation worldwide [18].

Major complications after SG include staple-line bleeding, gastric leak, stricture, GERD, and nutrient deficiency. Early and late complications were documented to be 0.7–5.8% and 1.2–10.8%, respectively. Reoperation rates range from 1 to 34% and also include those revisional bariatric procedures due to weight regain or severe GERD non-responsive to medical treatment [19].

18.2.4 Roux-en-Y Gastric Bypass

Roux-en-Y gastric bypass (RYGB) (Fig. 18.4) has been for long time the most popular bariatric-metabolic procedure worldwide and has been superseded only in recent years by SG.

RYGB involves the formation of a gastric pouch of approximately 30 mL which is anastomosed to the jejunum which is transected approximately 50–75 cm distal to

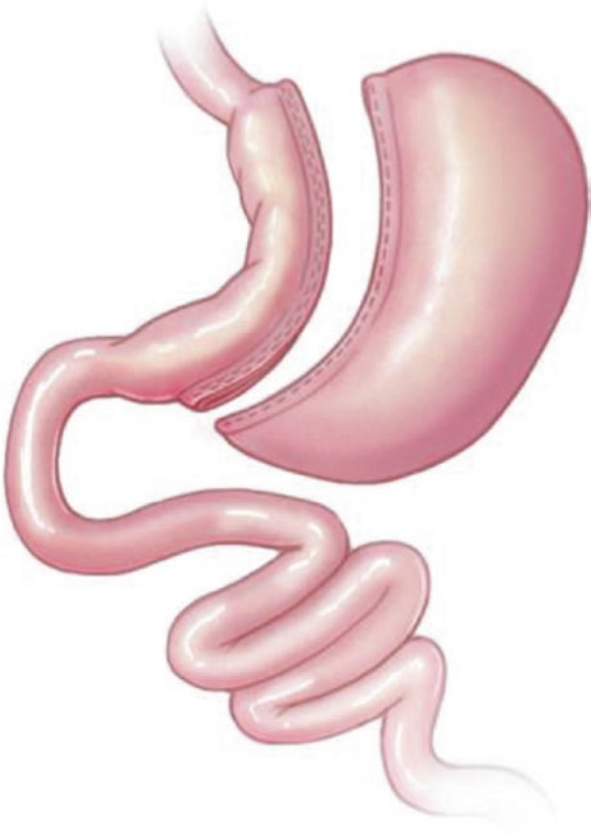

Fig. 18.3 Sleeve gastrectomy (SG) entails the longitudinal resection of the stomach along its greater curvature, carefully performing a complete excision of the gastric fundus, part of the body and antrum, yet maintaining a portion of the latter and the pylorus itself. The tube-shaped gastric sleeve maintains a capacity of approximately 60–150 mL

the ligament of Treitz (alimentary limb). The excluded gastric remnant in continuity with the duodenum and proximal jejunum represents the biliopancreatic limb, which is then connected to the alimentary channel through a jejuno-jejunostomy, 100–150 cm distal to the gastro-jejunostomy.

Early complications are stated to range from 4.8 to 9.4%, while late complications between 14.8 and 20.2% of cases. Reoperations are necessary in 2.5–38% of patients. Complications comprise anastomotic leaks, anastomotic strictures, marginal ulcers, internal hernia, dumping syndrome, and micronutrient deficiencies [19].

18.2.5 Biliopancreatic Diversion with Duodenal Switch

Initially projected by Scopinaro in 1979 [20], the biliopancreatic diversion (BPD) (Fig. 18.5) was consequently modified with the intention of reducing symptoms related to the "postgastrectomy syndrome" (i.e., nausea, vomiting, dumping syndrome, marginal ulcers). The duodenal switch (DS) adaptation of BPD was

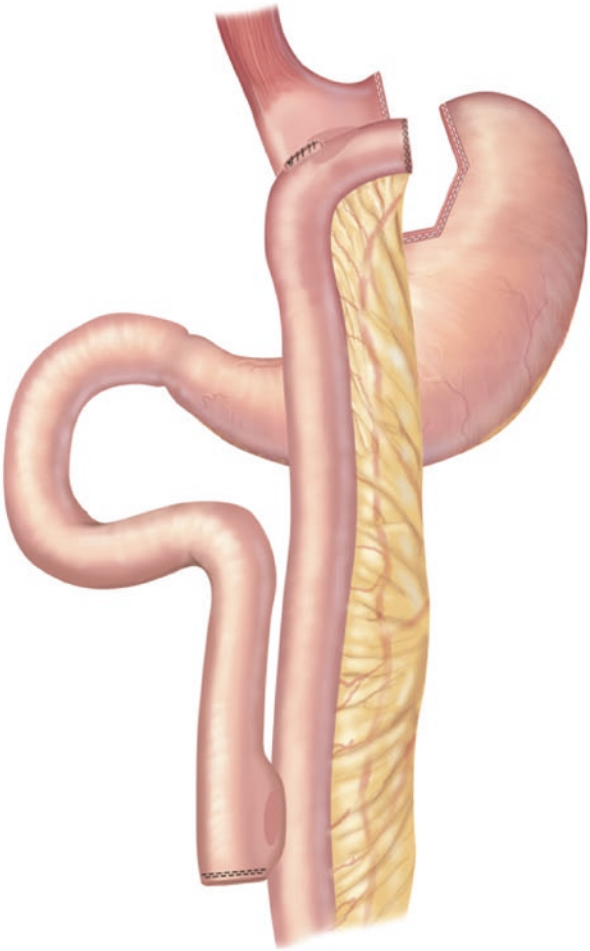

Fig. 18.4 Roux-en-Y gastric bypass (RYGB) involves the formation of a gastric pouch of approximately 30 mL which is anastomosed to the jejunum which is transected approximately 50–75 cm distal to the ligament of Treitz (alimentary limb). The excluded gastric remnant in continuity with the duodenum and proximal jejunum represents the biliopancreatic limb, which is then connected to the alimentary channel through a jejuno-jejunostomy, 100–150 cm distal to the gastro-jejunostomy. (With the kind permission of the New England Journal of Medicine (N Engl J Med 2012; 366:1577–1585))

described by Hess and Marceau [21, 22] aiming at the conservation of the pylorus which in turn contributes to reducing the risk of anastomotic marginal ulcers and dumping syndrome.

The first stage of the surgical procedure involves a SG which is then transected about 2 cm below the pylorus. The ileum is transected 250 cm cephalad to the ileo-cecal valve, and a duodenal-ileal anastomosis is created (alimentary limb). The biliopancreatic limb is anastomosed to the alimentary limb by an ileo-ileal anastomosis, 100 cm from the ileo-cecal valve, to form the common limb. This procedure comprises restrictive features to a greater malabsorptive component due to the short length of the alimentary tract, resulting in an elevated degree of nutrient malabsorption compared to other bariatric procedures. BPD-DS is also performed as a two-stage operation in order to reduce perioperative risk in super-obese patients (i.e., BMI >50 kg/m^2).

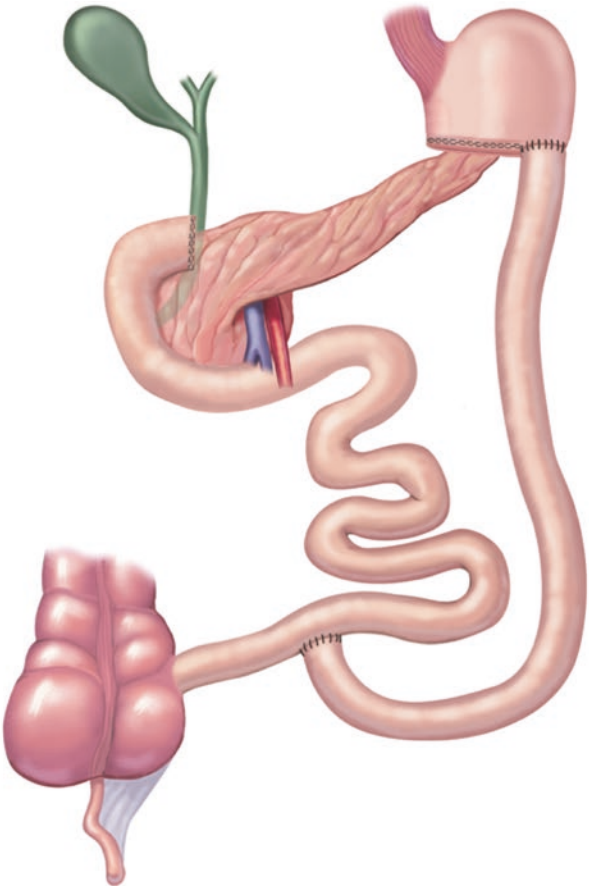

Fig. 18.5 Classic biliopancreatic diversion (BPD) consists of an about 60% distal gastric resection with stapled closure of the duodenal stump. The residual volume of the stomach is about 300 mL. The small bowel is transected at 2.5 m from the ileo-cecal valve, and its distal end is anastomosed to the remaining stomach. The proximal end of the ileum, comprising the remaining small bowel carrying the biliopancreatic juice and excluded from food transit, is anastomosed in an end-to-side fashion to the bowel 50 cm proximal to the ileo-caecal valve. Consequently, the total length of absorbing bowel is brought to 250 cm, the final 50 cm of which, the so-called common channel, represents the site where ingested food and biliopancreatic juices mix. (With the kind permission of the New England Journal of Medicine (N Engl J Med 2012; 366:1577–1585))

Early and late complications have been reported in 5.5–7.6 and 3.5–25.6%, respectively, while rate of reoperations is between 1.9 and 11.5%. BPD-DS complications include GERD, anastomotic or gastric leak, anastomotic stricture, internal hernia, severe malnutrition, nutrient deficiencies, increased bowel movements, and malodorous stools [19, 23, 24].

A modification of BPD-DS, the single-anastomosis duodeno-ileal switch (SADIS), has recently been introduced and is receiving growing attention. It has

demonstrated promising weight loss and comorbidity resolution rates. However, it is still at an early stage and no definitive conclusion can be made regarding its safety and effectiveness.

18.3 Bariatric Surgery and NASH: Clinical Trials

No data are reported in the literature regarding the effect of bariatric surgery on NASH in comparison to lifestyle modifications in randomized-controlled trials (RCT), rather the majority of the available information derive from small cohort studies.

Recently, the results of an RCT with primary outcome the weight loss deriving from RYGB or SG was analysed for NAFLD histological changes [9]. Liver biopsies were obtained during surgery in the whole cohort consisting of 66 subjects and liver function test performed at 1, 6, and 12 months after surgery; however, no histological data were obtained after surgery. About half of the subjects had histological diagnosis of NASH. At 1 year, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transpeptidase (γGT) were all significantly reduced. Circulating levels of albumin and INR were reduced after RYGB but not after SG, which may have implied some liver damage.

A prospective study including 109 subjects had 79% of patients' retention at 1 year [25]. This study demonstrated histological resolution of NASH in 85% of the subjects at 1 year after bariatric surgery that consisted in RYGB, bilio-intestinal bypass, or gastric banding. In another prospective study from the same authors [26], 381 liver biopsies were performed at baseline, 267 at 1 year and 215 at 5 years after surgery. The percentage of patients with NASH declined from 27.4 to 14.2% at 5 years. Steatosis and ballooning were drastically reduced at 1 year remaining stable at 5 years; instead, fibrosis worsened at 5 years although more than 95% of the patients had a fibrosis score ≤F1.

Very recently, a meta-analysis [27] including 15 retrospective and 17 prospective cohort studies with 3093 liver biopsies was published. Bariatric surgery determined biopsy-proven resolution of steatosis in 66%, inflammation in 50%, ballooning in 76%, and fibrosis in 40% of subjects. However, in 12% of subjects fibrosis worsened if present or appeared if absent at the baseline.

The effect of bariatric surgery on fibrosis is, however, controversial. In fact, in another study [28] involving 160 subjects with biopsies taken during the operation and between 6 months and 5 years after surgery, NASH was present at baseline in 27% of subjects with morbid obesity, who underwent bariatric surgery. NASH resolved in 90% of the cases. Overall, fibrosis resolved in 53% of the patients and improved in 3%, while grades 2 and 3 resolved in 60%.

A recent meta-analysis including 21 studies and 2374 patients shows that NASH improved in 59% and fibrosis in 30% of the patients [29]. Interestingly, the improvement of histological features was higher with a wedge than with a needle biopsy meaning that the type of biopsy is relevant for a correct diagnosis of NASH.

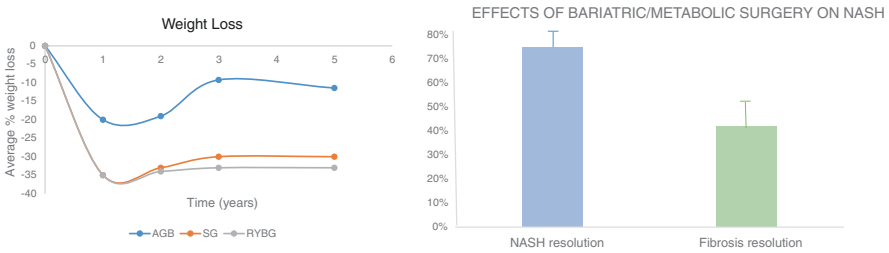

Fig. 18.6 Effects of bariatric/metabolic surgery on percent weight loss and on NASH and fibrosis resolution

At the moment, there are only two ongoing RCTs regarding the treatment of NASH with bariatric surgery. "A randomized controlled study on the effects of Roux-en-Y Gastric Bypass versus Sleeve Gastrectomy on Intensive Lifestyle Modifications on Non-Alcoholic Steato-Hepatitis," acronym BRAVES, on 288 obese subjects with NASH conducted at the Catholic University of Rome, Italy; ClinicalTrials.gov Identifier: NCT03524365.

The other RCT is "Vertical Sleeve Gastrectomy and Lifestyle Modification for the Treatment of Non-Alcoholic Steatohepatitis" conducted at the University of Minnesota—Clinical and Translational Science Institute on 60 participants. ClinicalTrials.gov Identifier: NCT03587831.

Few cases of hepatic failure have been described after biliopancreatic diversion with duodenal switch (BPD-DS) [30].

Figure 18.6 summarizes the effects of bariatric/metabolic surgery on percent weight loss and on NASH and fibrosis resolution.

18.4 Mechanisms of Action of Bariatric Surgery on NASH

Bariatric surgery has been renamed as "metabolic surgery" because some of its metabolic effects are independent of body weight reduction [4]. Metabolic surgery, in fact, dramatically improves insulin resistance just few days after BPD when the body weight was not significantly changed [31].

Metabolic surgery determines also type 2 diabetes remission with a weight-independent mechanism [32–36].

Forty-five milligrams of Pioglitazone daily for 6 months, a selective agonist for peroxisome proliferator-activated receptor-γ (PPAR-γ), in patients with type 2 diabetes or impaired glucose tolerance determined a significant improvement of histological and metabolic features of NASH, except for fibrosis, in comparison with placebo [37]. However, in another study with 30 mg of Pioglitazone per day fibrosis also improved [38].

In another RCT [39], 247 patients were randomized to one of the three arms: 30 mg of pioglitazone per day, 800 IU of Vitamin E per day, or placebo for 96 weeks. The primary outcome was an improvement in the NAFLD Activity Score (NAS)

compared with placebo with a significance of <0.025. Although pioglitazone did not reach the statistical threshold, a significantly greater proportion of patients receiving pioglitazone had complete resolution of NAS as compared with placebo: 47% versus 21% ($p = 0.001$).

A recent meta-analysis shows that pioglitazone therapy significantly reduced advanced fibrosis in liver biopsy either in subjects with diabetes or not [40].

Weight loss is effective to improve NAFLD. A 10% weight loss significantly decreased liver steatosis as assessed by CT scan in the Look Ahead Cohort [41]. Another study showed that 7% weight loss significantly improved steatosis, lobular inflammation, ballooning, and NAS, with minimal changes in fibrosis [42].

Since metabolic surgery is effective not only in reducing insulin resistance but also in determining massive weight loss, it is difficult to dissect the effects of each component on NASH. Interestingly, pioglitazone treatment has clearly demonstrated that histological improvement of NASH can be achieved even with a modest weight gain. Therefore, metabolic surgery could improve histological feature of NASH mainly through its action on insulin resistance.

At this regard, the literature is plenty of evidence that RYGB ameliorates hepatic insulin resistance [43–45] early after surgery, while BPD improves whole-body insulin sensitivity [46–48].

Undoubtedly, the conspicuous weight loss that accompanies metabolic surgery contributes to the improvement of hepatic liver features of NASH.

At 1 year, BPD/DS produces a significantly higher weight loss, of 19 kg on average, than RYGB and 35 kg more than lifestyle modifications. At 3 years, RYGB causes a weight reduction of 16.3 kg more than LAGB [49].

Seventy-two percent of patients who underwent RYGB had >20% weight loss, and 39.7% had >30% weight loss at 10 years compared with 10.8% and 3.9%, respectively, of nonsurgical matches [50]. Patients undergone RYGB lost 9.7% more of their baseline weight than patients who underwent SG; this difference was much higher as compared with LAGB (16.9%).

18.4.1 Microbiota After Bariatric/Metabolic Surgery and Its Effect on NASH

Lachnospiraceae bacterium and *Barnesiella intestinihominis* are two bacterial species which are abundant in stool of mice that developed NAFLD, while *Bacteroides vulgatus* is scarcely represented [51]. When transferred into germ-free mice, the former two species induce NAFLD [51]. *Gammaproteobacteria* and *Prevotella* are abundant in the feces of children with NAFLD as compared with children without NAFLD [52].

The relative abundance of the class *Gammaproteobacteria*, belonging to the phylum *Proteobacteria*, is increased in stool of subjects who underwent bariatric surgery as compared with lean controls [53–55]. Another abundant phylum after bariatric/metabolic surgery and, in particular, RYGB is the Verrucomicrobia, i.e., *Akkermansia muciniphila* [56].

The gut microbiota is modified in NAFLD/NASH as it is modified after bariatric/metabolic surgery; however, there are no prospective studies investigating the changes of gut microbiota after gastrointestinal surgery in comparison with lifestyle modifications that can help to clarify the role of intestinal flora in NAFLD/NASH.

18.5 Conclusions

Metabolic surgery is an effective treatment not only for NAFLD but also for NASH with resolution of the histologic features of NASH in more than 80% of the cases and disappearance of fibrosis in almost 50% of the patients. It is unclear, however, if the effects on NASH are due to weight loss or improvement of insulin resistance or both because all studies are conducted for at least 1 year after surgery when the reduction of weight is massive.

RCTs are needed to quantify with grade 1 evidence the efficacy and safety of metabolic surgery on NASH.

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Liver Transplantation and NAFLD/NASH

19

Damiano Patrono, Silvia Martini, and Renato Romagnoli

19.1 Nonalcoholic Fatty Liver Disease: An Indication for Liver Transplantation

As the global prevalence of nonalcoholic fatty liver disease (NAFLD) has raised up to 24%, being even higher in the Middle East (32%) and South America (31%), NAFLD and nonalcoholic steatohepatitis (NASH) are becoming increasingly common indications for liver transplantation (LT) worldwide [1, 2].

A study based on the Scientific Registry of Transplant Recipients analyzing data on 35,781 adult LT recipients from 2001 to 2009 identified NASH cirrhosis as the third indication for LT in the United States, behind hepatitis C-related cirrhosis and alcoholic liver disease; notably, NASH was the only indication with a progressively increasing trend over the study period [3]. Another study based on the United Network for Organ Sharing (UNOS) and Organ Procurement and Transplantation Network analyzing waitlist indications between 2004 and 2013 showed that NASH became the second most frequent indication for LT in 2013, with the steepest increase in terms of percentage (170% increase) [4]. Also among patients undergoing LT for hepatocellular carcinoma (HCC), analysis of UNOS data from 2002 to 2012 showed that NASH was the second leading etiology of cirrhosis behind chronic hepatitis C, with a fourfold increase in the number of patients transplanted for NASH-related HCC [5].

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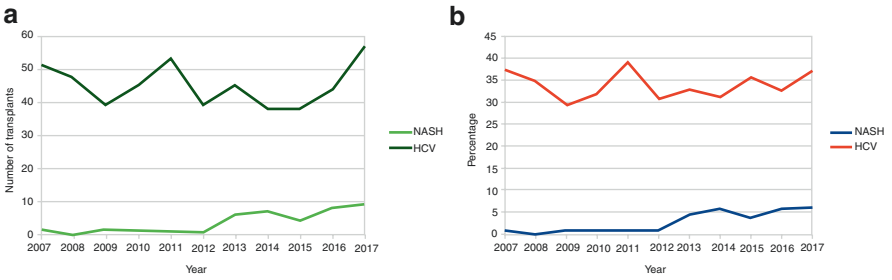

Fig. 19.1 Trend in indications for liver transplantation in the period 2007–2017 at our Institution: HCV versus NAFLD/NASH. Panel **a**: absolute number of liver transplants per year. Panel **b**: percentage of liver transplants per year

In Italy, the prevalence of NAFLD and NASH is similar. Data from the Italian National Institute of Health (Istituto Superiore di Sanità) report 35.5% of adult population being overweight and 9.8% being obese, meaning that 45.1% of subjects over the age of 18 suffer from an excess in body weight (source: www.epicentro.iss.it). Accordingly, at our Institution, we have observed a progressive increase in the absolute number (Fig. 19.1a) of LT performed for NASH-related cirrhosis, and the percentage has increased from less than 1% in 2007 to more than 5% in 2017 (Fig. 19.1b) (unpublished data).

This increase should be interpreted in the light of the fact that patients with NASH who are eventually transplanted likely represent a subpopulation of those who would actually benefit from a LT. Indeed, NASH and NAFLD are associated with an increased rate of cardiovascular disease, type 2 diabetes mellitus, obesity and renal function impairment, all of which can be considered as relative or absolute contraindications for LT, precluding access to the waiting list [6, 7]. In particular, obesity appears to play a major role in pre-LT assessment as it has been associated with increased duration of surgery and perioperative transfusion requirements, longer hospitalization in intensive therapy unit, increased rate of biliary and infectious complications, and decreased patient survival [8–10]. Once on the waiting list, NASH patients present higher mortality and increased drop-out rate as compared with other indications [3].

An interesting study by Segev et al. [11] based on UNOS data from 2002 to 2006 showed that obese patients already on the waiting list are less likely to get Model for End-stage Liver Disease (MELD) exception points and more likely to be turned down for an organ offer. Also, the chance of being transplanted was 11% and 29% lower in severely obese and morbidly obese patients, respectively, possibly reflecting the reluctance to transplant obese patients.

One reason for dropping out from the waiting list would also be the increased risk of portal vein thrombosis associated with NASH cirrhosis: in a study on 33,368 patients from the UNOS registry in the period 2003–2012, NASH as the etiology of liver disease was the strongest risk factor independently associated with portal vein thrombosis in patients undergoing LT (odds ratio 1.55; 95% confidence interval 1.33–1.81; $p < 0.001$) [12].

Liver transplantation for HCC in NAFLD and NASH also detains unique features. Patients with NASH-related HCC are characterized by older age at diagnosis and increased rate of comorbidities [13–15]. In an interesting study comparing the clinical pattern of HCC arising in NAFLD versus chronic hepatitis C, Piscaglia et al. [15] showed that HCC in the setting of NAFLD presented more frequently in a non-cirrhotic liver (46.2% versus 2.8%), and it was diagnosed more frequently outside a surveillance program. In the same study, HCC presented more frequently at an advanced stage in NAFLD than in chronic hepatitis C (Barcelona Clinic for Liver Cancer stage C rate 33.1% versus 23.9%, $p = 0.033$). This study confirms findings from several other studies showing an increased rate of HCC arising in a non-cirrhotic liver in the setting of NAFLD [13, 14, 16], which is linked to the independent cumulative effect of other risk factors for HCC like obesity and type 2 diabetes [17, 18]. As effective surveillance of HCC in patients with NAFLD is hampered by the overwhelming number of subjects at risk and by the lack of tools to stratify the risk of HCC [17], it is likely that a significant proportion of patients with NAFLD- and NASH-related HCC is diagnosed at an advanced stage and are therefore precluded the option for LT.

The challenge of LT in NAFLD/NASH appears therefore to be double: first, patients candidate to LT require more complex evaluation and pre-LT management due to increased age and comorbidities; second, prevention and treatment of comorbidities, as well as earlier diagnosis of HCC, would be necessary to extend provision of LT to those who are currently precluded this option.

19.2 Management Before Liver Transplantation

Patients candidate to LT for NASH require careful preoperative evaluation and possibly treatment or optimization of comorbidities before transplant.

19.2.1 Obesity and Overweight

As obesity is associated with worse outcome after LT [8–10], it has been identified as one potential target for patient optimization before transplantation. Weight loss, exercise, and dietary counseling are generally advised, but these are usually of limited practicability in patients with end-stage liver disease [19–21]. Dietary follow-up appears to be of paramount importance in NASH patients. If moderate and controlled weight loss is desirable, abrupt weight loss should be avoided as it may lead to further depletion of lean mass and developing/worsening of sarcopenia, which is defined as a significant loss of muscle and is a well-recognized risk factor for increased morbidity after LT.

In a study by Englesbe et al. [22] from the University of Michigan Medical Center, sarcopenia, being evaluated by measuring cross-sectional area of psoas muscle at the level of the fourth lumbar vertebra, was an independent prognostic factor of post-LT mortality: a decrease of total psoas area of 1000 mm^2 was associated with

a 3.7-fold increase of the risk of mortality. The same group highlighted also the impact of pre-LT transplant sarcopenia on the occurrence of severe infectious complications, which were in turn associated with reduced survival [23]. These findings have been confirmed also in the setting of living donor transplantation [24, 25], in which preoperative dietary interventions are easier to implement. To this regard, Kaido et al. [24] observed that preoperative administration of branched-chain amino acid supplements was associated with improved survival in sarcopenic patients. In another study, Masuda et al. [25] found that sarcopenia was associated with a two-fold increase in postoperative mortality and with a fivefold increased rate of sepsis; in this study, risk of sepsis was reduced by early administration of enteral nutrition.

In patients with NASH, overweight or obesity may be associated with muscle mass depletion, a condition known as sarcopenic obesity. The incidence and clinical relevance of sarcopenic obesity in LT candidates with NASH have been poorly studied. In a cohort of 207 patients from the University of Kentucky [26], sarcopenic obesity was observed in 38 subjects (18.4%) and was associated with a trend toward reduced 5-year patient survival; most notably, NASH-related cirrhosis (with a six-fold increased risk) and MELD score were the independent predictors of sarcopenic obesity. Thus, dietary counseling should have as a goal not only reduction of body weight but also preservation of lean mass.

Bariatric surgery before or during LT is an option to treat severe obesity. However, experience is limited to small case series. Among bariatric operations, sleeve gastrectomy is generally preferred as it does not cause malabsorption and should not alter the metabolism of immunosuppressants. In a first study by Takata et al. [27], 6 patients, who were precluded LT because of obesity, underwent laparoscopic sleeve gastrectomy with acceptable morbidity and no mortality. In another study by Lin et al. [28], 26 patients awaiting kidney transplantation ($n = 6$) or LT ($n = 20$, median MELD 11) underwent laparoscopic sleeve gastrectomy. In the whole series, the mean percentage of excess weight loss at 12 months was 50% and 7 patients eventually underwent LT ($n = 6$) or combined liver–kidney transplantation ($n = 1$). Although the results of these studies are encouraging, the major drawbacks of pre-LT bariatric surgery appear to be its limited applicability in patients with severely compromised liver function and the time needed to achieve significant weight loss. Thus, timing and indication for pre-LT bariatric surgery should be carefully discussed on a case-by-case basis.

Experience with bariatric surgery performed simultaneously with LT is also very limited. In a study by Heimbach et al. [29], 7 patients with Body Mass Index (BMI) > 35 kg/m^2 who underwent sleeve gastrectomy at the time of LT were compared with 37 patients who had LT alone after the desired weight loss was achieved with a dietary program. This study showed several interesting findings. First, a significant proportion (32%) of patients who achieved the target BMI (<35 kg/m^2) with diet were found to have BMI > 35 kg/m^2 at LT, highlighting the risk for weight regain and stressing the need for a close follow-up of these patients. Second, bariatric procedure had minimal impact on operative time and was generally well

tolerated, with only one patient developing a procedure-related complication (a leak from the gastric staple line). Third, no patient who underwent combined LT and sleeve gastrectomy suffered from post-LT diabetes (0% versus 34%, $p = 0.03$) or obesity (0% versus 60%, $p = 0.001$); one patient presented excess weight loss after combined operation requiring dietary counseling. Placement of an adjustable gastric banding during LT has also been reported, resulting in 45% excess weight loss and improvement in metabolic syndrome comorbidities [30]. Further data are necessary to evaluate the feasibility of a wider application of this approach. Anyway, the decision of considering bariatric surgery simultaneously with LT should take into account the degree of pre-LT hepatic insufficiency, nutritional status, weight excess, patient comorbidities, and expected waitlist time.

19.2.2 Cardiac Function

Assessment of cardiac function is of paramount importance as NASH is frequently associated with several risk factors for cardiovascular disease like obesity, arterial hypertension, diabetes, and dyslipidemia. It is also not clear whether NASH and NAFLD, independently from associated comorbidities, are associated per se with cardiovascular disease due to the systemic inflammatory imbalance [31]. In a study by Patel et al. [32] on 420 patients (125 alcohol-related and 295 nonalcohol-related end-stage liver disease) assessed before LT, the incidence of severe coronary artery disease (>70% diameter stenosis) was 13% in the nonalcohol-related group versus 2% in the alcohol-related group ($p < 0.005$); the absence of cardiac risk factors was highly predictive of the absence of significant coronary artery disease, while dobutamine stress echocardiography had a poor predictive value of significant coronary artery disease but was useful in ruling out patient without significant disease.

In another study by Vanwagner et al. [33], patients with NASH, even after adjusting for potential confounders, had a fourfold increase in the risk of experiencing adverse cardiovascular events during the first year after LT. Treating coronary artery disease pre-LT appears to be worthwhile as treated patients have comparable survival after LT as compared to control group [34]. As obesity is associated with porto-pulmonary hypertension, NASH patients should be adequately screened. In case a suspicion of porto-pulmonary hypertension is raised at trans-thoracic echocardiography, right heart catheterization is indicated; if a moderate or severe pulmonary hypertension is confirmed, patients should be started on appropriate treatment [35]. Clinical LT guidelines from the European Association for the Study of the Liver (EASL) recommend cardiopulmonary exercise test in patients older than 50 years or with multiple associated cardiovascular risk factors, reserving coronary angiography to patients in whom coronary artery disease is suspected [36]. As of today, to the best of our knowledge, there are no specific guidelines for cardiac evaluation in candidates to LT with NASH. Whether EASL guidelines should be modified in this specific subset of patients is matter of debate.

19.2.3 Renal Function

Candidates to LT with NASH seem to have an increased risk of pre- and post-LT renal dysfunction as compared to other etiologies of end-stage liver disease. In a study by Park et al. [37] based on 569 patients referred at the Transplant Institute at Hawaii Medical Center—East over a 10-year period, NASH patients had a higher creatinine level (1.26 mg/dL versus 0.98 mg/dL; $p = 0.0018$) as compared to other etiologies; NASH and cardiac disease were independent predictors of serum creatinine level. The study by Singal et al. [38] analyzing the indications for combined liver–kidney transplantation in the UNOS database from 2002 to 2011 showed a 2.5-fold increase in the percentage of combined transplants performed in NASH patients, whereas in chronic hepatitis C patients the rate decreased from 44 to 34%. The same study showed reduced patient, kidney graft, liver graft survival, and poorer renal function in patients transplanted for NASH, HCV, and HCC [38]. While worse outcome could be attributed to HCV recurrence in pre-direct acting antivirals era and to HCC recurrence, the same finding is worrisome in NASH patients, in whom poorer outcome and renal function could be explained by the burden of comorbidities impacting on both survival and renal function.

Indications for combined liver–kidney transplantation in NASH patients should follow those in general LT candidates, i.e., (1) end-stage liver disease and glomerular filtration rate less than 30 mL/min; (2) hepato-renal syndrome requiring renal replacement therapy more than 8–12 weeks; (3) more than 30% fibrosis and glomerulosclerosis at renal biopsy [36, 39]. The indication for combined liver–kidney transplantation in patients with creatinine clearance between 30 and 60 mL/min should be weighed against the availability of kidney grafts and the risk of long-term degradation of renal function [36]. Careful pre-LT evaluation, tailored immunosuppression, and timely management of comorbidities with a potential detrimental effect on renal function are therefore of significant relevance in NASH patients.

19.2.4 Diabetes

As diabetes is a feature of metabolic syndrome, NASH patients referred for LT frequently present with type 2 diabetes. This could impact on post-LT outcome through diabetes complications, in particular cardiovascular disease. The independent effect of diabetes on survival and other outcomes after LT has been poorly studied. Yoo et al. [40] studied the influence of diabetes on LT outcome in a cohort of 21,391 patients from the UNOS database. In this study, 5-year patient (63.4% versus 75.0%, $p < 0.001$) and graft (56.7% versus 67.0%, $p < 0.001$) survival were lower in patients with type 1 diabetes compared with those without diabetes; type 2 diabetes was associated with decreased graft survival only. Coronary artery disease was another independent predictor of lower survival and its association with diabetes showed an additive effect in reducing survival. In a single-center study from the University of North Carolina School of Medicine to assess the independent effect of NAFLD and associated comorbidities on survival after LT, NAFLD was associated

with reduced 30-day survival, whereas diabetes was associated with higher 3-year mortality (HR 3.58, CI 1.32, 9.71; $p = 0.01$) [41]. Diabetic patients, even more in the setting of NAFLD/NASH, require thus careful evaluation and optimization of glycemic control. In some cases, end-stage diabetes complications (as complicated peripheral vasculopathy, neuropathy, or retinopathy) may represent a contraindication to LT, according to center practice.

19.3 Outcomes of Liver Transplantation in NAFLD/NASH

Due to the burden of comorbidities and the increased surgical risk associated with obesity, LT in patients with NAFLD/NASH is considered to be challenging as compared to other common indications. Thus, it would not be surprising that patients transplanted for NASH cirrhosis had worse outcome than those transplanted for other indications, rising questions about benefit and sustainability of LT performed in this setting.

Outcomes of LT for NASH have been investigated in several studies (Table 19.1). First single-center studies comparing patients transplanted for NASH with other

Table 19.1 Survival outcomes in patients transplanted for NASH cirrhosis

Author, Journal, Year	Study period	Study design	Survival outcome	Observations
Malik et al. Am J Transplant 2009	1997–2008	Single-center 98 NASH patients compared to cholestatic ($n = 196$), alcoholic ($n = 196$), HCV-related ($n = 196$), or cryptogenic ($n = 98$) cirrhosis	Comparable 5-year survival (72.4% vs. 65.3–81.6%)	Sepsis was most frequent cause of death in NASH patients. Poor outcome in NASH group in association with age ≥60 years and BMI ≥ 30 kg/m^2
Bhagat et al. Liver Transpl 2009	1997–2007	Single-center 71 NASH patients compared to 83 with alcoholic cirrhosis	Comparable 5- (75% vs. 86%) and 9-year (62% vs. 76%) survival	Sepsis was the most frequent cause of death in both groups, followed by cardiovascular events in NASH
Barrit et al. J Clin Gastroenterol 2011	2004–2007	Single-center 21 NASH compared to 97 other indications	Lower 30-day (81% vs. 97%) but comparable 3-year (76% vs. 84%) survival	No differences in causes of death between NASH and non-NASH patients
Park et al. Clin Transplant 2011	1998–2008	Single-center 71 NASH compared to 472 other indications	Comparable 2-year survival (78% vs. 84.6%)	NASH patients had comparable MELD score at LT but worse renal function

(continued)

Table 19.1 (continued)

Author, Journal, Year	Study period	Study design	Survival outcome	Observations
Charlton et al. Gastroenterology 2011	2001–2009	Registry (Scientific Registry of Transplant Recipients data) 1959 NASH compared to 33,822 other indications	Comparable 1- (84% vs. 87%) and 3-year (78% vs. 78%) survival	Patient and graft survival for NASH patients was similar to that for other indications
Agopian et al. Ann Surg 2012	1993–2011	Single-center 144 NASH patients compared to HCV-related (n = 691), HBV-related (n = 127), alcoholic (n = 185), cryptogenic (n = 58), and cholestatic (n = 89) cirrhosis	Comparable 5-year survival (70% vs. 63–70%); better survival than HCV patients (70% vs. 54%)	BMI > 35 kg/m^2 was associated with worse survival only in NASH patients; pre-LT dialysis was the strongest predictor of lower survival
Kennedy HPB (Oxford) 2012	1999–2009	Single-center 129 NASH patients compared to 775 other indications	Comparable 5-year survival (85% vs. 80%)	High-risk phenotype (age > 60 years, BMI > 30 kg/m^2, hypertension and diabetes) was associated with lower survival
VanWagner et al. Hepatology 2012	1993–2010	Single-center 115 NASH patients compared to 127 alcoholic liver disease	Comparable 3- (73.3% vs. 85.3%) and 5-year (60.3% vs. 68.8%) survival	NASH patients were more likely to experience an adverse cardiovascular event during first year after LT
Afzali et al. Liver Transpl 2012	1997–2010	Registry (UNOS data) 1810 NASH patients compared to 51,928 other indications	Comparable 3- (82.2% vs. 75.2%–88%) and 5-year (76.7% vs. 66.7%–84.2%) survival	NASH patients had a higher risk of cardiovascular events and a lower risk of graft loss
Thuluvath et al. Transplantation 2018	2002–2016	Registry (UNOS data) 4089 NASH compared to cryptogenic (n = 3241), alcoholic (n = 7837) or autoimmune (n = 1435) cirrhosis	Comparable 5- (77% vs. 77–79%) and 10-year (63% vs. 60–65%) survival	Patient and graft survival for NASH patients were similar to that for cryptogenic, alcoholic, or autoimmune cirrhosis

NASH nonalcoholic steatohepatitis, *HCV* hepatitis C virus, *BMI* body mass index, *LT* liver transplantation, *HBV* hepatitis B virus, *UNOS* United Network for Organ Sharing, *MELD* Model for End-stage Liver Disease

etiologies showed an increased risk of early mortality and cardiovascular events, but comparable long-term survival [41–43]. Agopian et al. [44] analyzed 144 adult NASH patients who underwent LT between December 1993 and August 2011 at the Dumont-UCLA Transplant Center. Over the study period, the frequency of LT for NASH increased fivefold and despite comparable long-term outcome, NASH patients had significantly longer operative times and postoperative length of stay, as well as higher intra-operative blood losses. In 2014, Wang et al. [45] performed a meta-analysis combining data across 9 studies [33, 37, 41–44, 46–48]. Patients transplanted for NASH showed comparable survival, an increased rate of death due to sepsis and cardiovascular events, but a lower rate of death due to graft loss, suggesting that excellent outcome can be achieved, provided that early complications are prevented or managed.

The finding of a comparable long-term post-LT survival of NASH patients, along with the increasing rate of transplants performed for this indication, was confirmed by two large registry studies based on the UNOS and Scientific Registry of Transplant Recipients (SRTR), respectively [3, 49]. Afzali et al. [49] analyzed from the UNOS database 53,738 patients who underwent LT between January 1997 and October 2010. Survival of NASH patients at 1, 3 and 5 years after LT was inferior only to that of patients transplanted for end-stage liver disease secondary to primary biliary cholangitis, primary sclerosing cholangitis, autoimmune hepatitis, or chronic hepatitis B. Charlton et al. [3], analyzing SRTR data on 35,781 primary adult LT recipients in the period between January 2001 and December 2009, showed that patients transplanted for NASH, despite being older, having greater BMI and lower HCC rate, had comparable 1- and 3-year survival of patients transplanted for other indications, even after adjusting for sex, BMI, age, and serum creatinine level. It is possible that results from these two studies were partially flawed by a missing diagnosis of NASH in approximately a half of patients who were coded as having cryptogenic cirrhosis. In a more recent study, Thuluvath et al. [50] analyzed the UNOS data on LT recipients from February 2002 to September 2016 and compared the outcomes of LT for cryptogenic cirrhosis, autoimmune hepatitis, NASH, and alcoholic liver disease. No significant differences were found among different indications. In this study, NASH patients had the lowest rate of graft loss, whereas older age, male sex, severe disability (defined as Karnofsky performance score $\leq 30\%$), presence of hepatic encephalopathy, portal vein thrombosis, and being on dialysis pretransplant negatively impacted survival.

Overall, according to the aforementioned studies, it seems that survival outcomes after LT for patients with NASH-related cirrhosis are at least as good as for those with other indications although NASH patients tend to have an increased rate of early post-LT mortality due to cardiovascular accidents and infectious complications. However, two considerations need to be done. First, these good outcomes could have been achieved by selecting only fitter patients, thereby excluding a significant proportion of potential candidates due to comorbidities or other reasons. The rate of LTs performed for NASH is growing steeply, but the absolute number of transplants performed for this indication remains minimal when compared to the global epidemic and to the number of potential candidates, suggesting that the

impact of LT on the burden of NASH in the general population is still quite limited. Second, most published registry and single-center studies included a significant proportion of patients transplanted for chronic hepatitis C-related cirrhosis in the interferon era. Direct-acting antivirals have been introduced in the last years, and thanks to their high efficacy and tolerability, they have revolutionized HCV therapy and are going to change also the landscape of indications for LT. As a consequence, survival outcomes after LT in HCV patients (and, consequently, in the whole LT recipients' population) are significantly improving [51], setting a new benchmark against which outcomes of LT in patients with NASH will have to be compared.

19.4 Recurrent and de novo NAFLD/NASH After Liver Transplantation

The observation of a trend toward an excess weight gain after LT is quite common among physicians involved in the follow-up of LT recipients. According to the literature, approximately 50–60% of overall LT recipients present features of metabolic syndrome and insulin resistance [31, 52]. Immunosuppressive drugs play a role in favoring the worsening or development of metabolic syndrome: steroids favor gluconeogenesis, weight gain, and insulin resistance; calcineurin inhibitors (especially tacrolimus) are diabetogenic, whereas mTOR inhibitors are associated with dyslipidemia. However, the burden and clinical consequences of recurrent or de novo NAFLD and NASH after LT have been investigated only in few studies.

The rate of recurrent NASH after LT was first investigated in a study from the University of Miami comparing patients transplanted for NASH-related cirrhosis with those transplanted for alcoholic liver disease [42]. In this study, recurrent NASH was observed in 33% of the patients, whereas no patient transplanted for alcoholic cirrhosis developed NASH ($p < 0.001$); however, in the recurrent NASH group none developed NASH-related cirrhosis or needed re-LT. Notably this study showed a higher incidence of acute or chronic rejection in the NASH group (45% versus 25%; $p = 0.011$), despite comparable survival [42]. In subsequent studies, the rate of allograft steatosis after LT varies between 30 and 100%, with patients transplanted for NASH exhibiting higher risk; however, recurrent NASH or NAFLD did not negatively affect overall survival [53].

Another study from the University of Pittsburgh [54] evaluated the incidence of NAFLD, NASH, and the progression of fibrosis in patients transplanted for NASH ($n = 77$), who were compared with patients transplanted for alcoholic liver disease ($n = 108$). At 1-year follow-up, more than 50% had graft steatosis; in NASH patients, 16% had moderate/severe steatosis, 6.8% had NASH, and 2.3% had advanced fibrosis at 1 year, with no difference between the two groups. About 20% of the patients had a liver biopsy at fifth-year follow-up: in the NASH patients, 35% had moderate/severe steatosis, 30% had NASH and 5.9% advanced fibrosis, with no difference between NASH and alcohol groups. However, a significantly higher rate of patients transplanted for alcoholic liver disease developed cirrhosis over the study period (9.3% vs. 0%, $p = 0.0075$). Recently, Bhati et al. [55] from Virginia Commonwealth

University, Richmond, VA, evaluated by liver biopsy and transient elastography, the rate of recurrent NAFLD, NASH, and fibrosis in patients transplanted for NASH or cryptogenic cirrhosis with features of metabolic syndrome. In the 34 patients who had a liver biopsy at a median time from LT of 47 months, NAFLD recurred in 88.2% and NASH in 41.2%; 20.6% had bridging fibrosis but only one developed clinical signs of portal hypertension and decompensated cirrhosis [55].

From the aforementioned studies, it appears that although recurrence of NAFLD and NASH is quite common after LT for NASH, progression to clinically significant fibrosis is rarely observed.

The incidence and impact of de novo NAFLD and NASH in patients transplanted for indications other than NASH represent another side of the question. In a single-center study by Seo et al. [56], incidence of de novo NAFLD and NASH after LT was 18% and 9%, respectively. A 10% BMI increase after LT was associated with higher risk of developing de novo NAFLD, whereas treatment with angiotensin-converting enzyme inhibitors was associated with lower risk. In a 2017 meta-analysis by Losurdo et al. [57], 12 studies were selected, enrolling 2166 patients: pooled weighted prevalence of de novo NAFLD and NASH was 26% and 2%, respectively. Patients transplanted for alcoholic and cryptogenic cirrhosis had the highest risk of de novo NAFLD, whereas there was no difference in NAFLD recurrence between patients treated with tacrolimus or cyclosporine. A recent study by Narayanan et al. [58] investigated the prevalence of de novo graft steatosis in a cohort of 588 patients transplanted in the period 1999–2006 (9.4% NASH recipients). Overall, the prevalence of graft steatosis at 10-year follow-up was 43.2% and was higher in patients transplanted for NASH cirrhosis (77.6%). Risk factors for graft steatosis were female sex, LT for chronic hepatitis C, and time-dependent BMI. Graft steatosis was not associated with decreased graft survival or with an increased risk of cardiovascular accidents, whereas underlying NASH was associated with a twofold increase of cardiovascular risk [58].

It has also been suggested that, although clinical picture and histologic features may be quite similar, recurrent and de novo NAFLD/NASH have different severity and clinical implications. In a single-center study by Vallin et al. [59], 11 patients with recurrent NAFLD were compared with 80 patients with de novo NAFLD. Severe fibrosis and steatohepatitis were more frequent in patients with recurrent NAFLD versus patients with de novo NAFLD, suggesting that recurrent NAFLD would be a more aggressive disease as compared to de novo NAFLD. This would be in keeping with evidence from population-based [60, 61] and genetic studies [62, 63] of a genetic background predisposing to fat accumulation in the liver and to progression toward fibrosis and cirrhosis [64].

Prevention of recurrent and de novo NAFLD and NASH after LT remains a matter of debate. As of today, there is no effective drug against these entities. Clinical interventions like dietary counseling to limit weight gain, promotion of physical exercise, and tailoring immunosuppression are likely to be beneficial but need prospective validation [31]. Bariatric surgery after LT has been occasionally reported [65]. Compared to bariatric surgery performed pre-transplant or simultaneously with LT, this approach has the advantage to select patients with stable hepatic

function and persistent obesity. However, the operation could be technically more demanding due to previous LT and adhesions, which may represent a contraindication to the laparoscopic approach. In total 22 cases of bariatric surgery after LT have been reported (sleeve gastrectomy, $n = 11$; Roux-*en*-Y gastric by-pass, $n = 10$; biliopancreatic diversion, $n = 1$) with no postoperative mortality, but increased morbidity as compared to the general population [65]. Due to the very limited available experience, this approach needs to be evaluated in larger series.

19.5 Donor Implications

As characteristics of donor pool reflect those of reference population, NAFLD has become more and more frequent also among organ donors. Over last 2 years, at our Institution median donor age was 65 years (interquartile range: 50–76) and median BMI was 25.3 kg/m^2 (interquartile range: 22.9–27.7); 66.1%, 18.2%, and 3.2% of donors were overweight (BMI $\geq$ 25 kg/m^2), obese (BMI $\geq$ 30 kg/m^2), or severely obese (BMI $\geq$ 35 kg/m^2), respectively; 13.6% and 4% of the grafts showed a macrovesicular steatosis $\geq$15% and $\geq$30%, respectively.

It is well-known that steatotic grafts suffer from a more severe degree of ischemia-reperfusion injury. This is primarily related to reduced ATP content that causes a shift in the mechanism of cell death from apoptosis (which is an energy-dependent process) to necrosis, with consequent formation of an inflammatory milieu [66, 67]. Impaired baseline microcirculation and disturbances in hepatic flow after reperfusion also worsen ischemia-reperfusion injury. This is caused by increased susceptibility of sinusoidal endothelial cells to ischemia-reperfusion injury, leading to increased expression of adhesion molecules, inflammation, vasoconstriction, and obliteration, a phenomenon that has been observed in both experimental [68–70] and clinical setting [71].

Use of steatotic grafts has been associated with an increased rate of primary non-function, early allograft dysfunction (as defined by Olthoff et al. [72]), reduced patient and graft survival, longer intensive therapy unit and hospital length of stay, and increased costs [73, 74]. Therefore, graft steatosis along with body weight and BMI (which are strongly correlated) has been incorporated in several prognostic scores of patient and graft survival after LT [75–78].

Some authors advocate that disturbances of microcirculation could impair also peri-biliary plexus perfusion, leading to an increased rate of biliary complications as liver graft steatosis has also been identified as a risk factor for biliary complications [79]. The degree and type (macrovesicular versus microvesicular) of steatosis may also play a role. While it is generally accepted that grafts with mild (<30%) macrovesicular steatosis can be used safely, use of grafts with moderate (30–60%) steatosis is more controversial and grafts with severe (>60%) steatosis are generally discarded. On the other hand, the degree of microvesicular steatosis seems to be somewhat less important with regard to LT outcomes [80].

Unfortunately, due to the ever-increasing disparity between demand and organ availability, simply discarding steatotic grafts does not appear to be a practicable

attitude. In 2017, 1296 liver transplants were performed in Italy; however, at the end of the year there were still 991 patients on the waiting list, 89 died before having the opportunity to get a transplant, and another 55 were withdrawn from the list for other reasons (source: www.trapianti.salute.gov). Therefore, effective means to optimize LT outcome using steatotic grafts are urgently needed.

Classical strategy has been to minimize possible other risk factors by matching these high-risk donors with low-risk recipients and/or reducing ischemia time [81]. In a 2016 study from the Hong Kong group [82], use of severely steatotic grafts with >60% macrovesicular steatosis was associated with short- and long-term survival comparable to those of control group; notably, authors observed no case of primary non-function nor early allograft dysfunction, advocating that short ischemia time (median 384 min) and prudent allocation (median MELD score = 20) were key factors in achieving such good outcomes. In our experience [83], despite short ischemia time, use of grafts with >60% steatosis was associated with a 20% rate of primary non-function requiring urgent re-LT, and a 90% rate of early allograft dysfunction; more importantly, the incidence of postoperative renal failure was 90%, and 6 out of 9 recipients required renal replacement therapy. It therefore appears that although good long-term results can be achieved, fatty grafts require additional treatment to optimize early outcome after LT. Ischemic and pharmacological preconditioning of liver grafts, although promising, has not gained wide clinical acceptance [84, 85].

Currently, the main area of research concerning preservation of grafts from the so-called extended criteria donors, including steatotic grafts, is machine perfusion. After the pioneering works of Jim Guarrera group at the Columbia University [86–88], a renewed interest has grown concerning the potential benefits of dynamic perfusion techniques as compared to classical static cold storage. Both hypothermic oxygenated and normothermic perfusion have proven to be feasible in the clinical setting and effective in improving organ preservation and reducing ischemia-reperfusion injury [89–91]. In the context of graft steatosis, experimental data show that hypothermic [92] or subnormothermic [93–95] oxygenated perfusion are associated with improved histology, bile production, hepatic flows, and liver function as compared to static cold storage (Table 19.2). Also, fatty grafts treated with oxygenated perfusion exhibited reduced levels of high-mobility group box protein-1 and decreased lipid peroxidation, but increased tissue content of adenosine triphosphate and glutathione [94]. A recent study by Kron et al. [96] confirmed these findings by showing reduced downstream activation of Kupffer cells by toll-like receptor-4, cytokine release, and endothelial activation in a rat model of liver steatosis induced in rats by methionine-choline-deficient diet. In the same study, outcomes of 6 human LTs using steatotic grafts (of which 5 from donor after cardiac death) were compared with those of 12 matched cases: recipients of grafts treated with 1-h end-ischemic oxygenated machine perfusion showed lower rate of primary non-function, reduced need for renal replacement therapy, shorter length of stay in intensive therapy unit, and improved patient survival [96]. To date, this is the only study confirming the value of machine perfusion in LT with steatotic grafts. Using hypothermic oxygenated perfusion, Monbaliu et al. [97] observed that perfusate levels of aspartate aminotransferase and lactate dehydrogenase correlate with graft quality, proposing

Table 19.2 Experimental studies investigating machine perfusion for preservation of steatotic grafts in liver transplantation

Author, Journal, Year	Methods	Findings
Bessems et al. Liver Transpl 2007	Fatty rat liver grafts (choline-methionine-deficient diet inducing 30–60% steatosis) preserved over 24 h were evaluated by isolated-perfused rat liver model	Bile production, ammonia clearance, urea production, oxygen consumption, and ATP levels were significantly higher after MP as compared to CS. Machine perfusion improved preservation
Vairetti et al. Liver Transpl 2009	Zucker rat livers were exposed to 6-h cold storage or oxygenated machine perfusion at different temperatures (4, 8, and 20 °C) and then reperfused over 2 h. A modified Krebs-Henseleit solution was used for cold, subnormothermic, and warm reperfusion	Subnormothermic MP improved preservation as demonstrated by lower AST and LDH release, improved ATP/ADP ratio, decreased Caspase-3 activity, improved bile production, lower portal pressure, and better histology. Authors hypothesize protection of mitochondria could explain better preservation
Boncompagni et al. Eur J Histochem 2011	Lean and fatty Zucker rat livers were exposed to 6-h cold storage or subnormothermic (20 °C) oxygenated machine perfusion and then reperfused over 2 h at 37 °C	In fatty livers, subnormothermic MP was associated with lower portal pressure during normothermic perfusion and lower degree of hepatocytes and sinusoidal cells apoptosis
Monbaliu et al. Liver Transpl 2012	Seventeen discarded human liver were perfused with a modified LifePort kidney circuit over 24 h with non-oxygenated UW-MP solution. Eleven livers that were judged non-transplantable were compared with six that would have been potentially transplantable	Authors observed higher AST and LDH release in perfusate of non-transplantable livers. Degree of macrovesicular steatosis correlated well with AST release. Perfusate analysis showed a progressive decrease in pH, oxygen consumption, CO_2 accumulation, and lactate increase. There were no differences in machine flows between two groups, as well as in perfusate cytokines level
Okamura et al. Am J Transplant 2017	Severe steatotic grafts preserved by either cold storage or subnormothermic oxygenated MP for 4 h using polysol solution, then evaluated during 2 h during normothermic perfusion	Tissue ALT and mitochondrial glutamate dehydrogenase were reduced after subnormothermic MP. Portal venous pressure, tissue adenosine triphosphate, bile production, high-mobility group box protein-1, lipid peroxidation, and tissue glutathione were all significantly improved by subnormothermic MP

Table 19.2 (continued)

Author, Journal, Year	Methods	Findings
Kron et al. J Hepatol 2017	– Experimental study in which hepatic steatosis was induced in rats by methionine-choline-deficient diet. After 12 h of cold storage, livers were transplanted directly or after 1-h hypothermic oxygenated machine perfusion (HOPE) or hypothermic machine perfusion with nitrogen – Clinical report about 6 human steatotic grafts (5 retrieved from donors after cardiac death) transplanted after HOPE, which were compared to 12 steatotic grafts transplanted after static cold storage, matched for donor and recipient age and total preservation time	– In the experimental study, HOPE improved significantly reperfusion injury, as demonstrated by reduced downstream activation of Kupffer cells by TLR-4, cytokine release, and endothelial activation. Liver function tests and animal survival were also improved. Substitution of oxygen with nitrogen suppressed HOPE beneficial effect – In the group of patients transplanted after static cold storage, authors observed significantly increased primary non-function rate, need for renal replacement therapy, longer intensive care unit stay, and lower patient survival

ATP adenosine triphosphate, *MP* machine perfusion, *CS* cold storage, *AST* aspartate aminotransferase, *ADP* adenosine diphosphate, *LDH* lactate dehydrogenase, *UW-MP* University of Wisconsin solution for machine perfusion, *ALT* alanine aminotransferase, *HOPE* hypothermic oxygenated machine perfusion, *TLR-4* toll-like receptor 4

this could be a way to assess graft function preoperatively. However, normothermic machine perfusion, by exposing the organ to an almost physiological environment, appears to be a much more powerful tool to assess pre-transplant organ function [98]. As of today, there are at least 12 trials actively recruiting patients to evaluate the impact of different machine perfusion techniques in various clinical settings (source: https://clinicaltrials.gov/). The promises of machine perfusion are numerous, including providing better preservation, reducing ischemia-reperfusion injury, allowing objective assessment of organ quality before transplantation (thereby avoiding futile or potentially harmful transplants), and serving as a platform for pharmacologic or biologic intervention to improve graft quality before implant [99–101]. Whether these promises will be fulfilled, also in regard to the specific setting of fatty liver grafts, has yet to be determined.

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