NAFLD and NASH

Biomarkers in Detection, Diagnosis and Monitoring

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Foreword

This book addresses current state of the art on the development of biomarkers in different scenarios of the disease: general population and primary care, in pediatric patients, and in patients with chronic liver diseases at risk of progression to cirrhosis, liver cancer and extrahepatic outcomes. Biochemical biomarkers including routine methods and omics from genetic to proteomic and metabolomic and imaging biomarkers from ultrasonography and transient elastography to magnetic resonance have been addressed focusing on clinical utility.

Authors from all over the world (Spain, Israel, Australia, Italy, Switzerland, the United Kingdom, Greece, Brazil, and Chile) highly motivated and expert on NAFLD participated enthusiastically in this book.

This work includes the thoughts and knowledge of the authors together with the current evidence available. In this evolving area, the performance of this work should be useful for the decision-making process in clinical practice of physicians working on all the levels of Health System.

We hope you enjoy reading it. Thank you.

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Introduction to Biomarkers Development

The development of biomarkers to diagnose and monitor progression and treatment response in NAFLD is a major challenge in medical community. NAFLD is a systemic and multi-axis disease that provides essential elements participating on its pathophysiology that play a crucial role on the scenario of developing biomarkers. The intense relationship with many other organs like gut, kidney, pancreas, or brain together with the role of alterations in different axis like gut-liver axis, brain-liver axis, kidney-liver axis, and the metabolic-liver axis allowed us to look for many biomarkers.

A biomarker could be defined as a characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions. We could define biomarkers for susceptibility, diagnosis, monitoring, and prognosis together with predictive response to therapy and safety. The main characteristics of a biomarker should be: (a) availability and acceptability, (b) lack of bias of process, (c) cost-effectiveness, (d) high diagnostic accuracy, (e) errors measurement, and (f) reliability. Three aspects measuring validity are: (1) content validity, (makes sense) a biomarker reflects the biological phenomenon studied; (2) construct validity, (algorithm) in the network of biomarkers or disease manifestations; (3) criterion validity, how the biomarker correlates with the specific disease and is usually measured by sensitivity, specificity, and predictive power.

In the development of biomarkers, liver biopsy analysis has been utilized as gold standard. Interpretation of liver biopsy specimens showed several limitations including: (a) weak concordance between pathologists in the diagnosis of several types of lesions, mainly steatohepatitis (inflammation and ballooning); (b) overlap between features. It is very common that cases with higher inflammation showed advanced fibrosis, but not in all cases; (c) sample size limitations of liver specimens due to heterogeneous distribution of lesions in the liver is common; (d) progression over the time and dynamic nature of this entity with progression and regression episodes that preclude a unique liver biopsy could correctly stratify the risk of the patient, at least in cases without significant fibrosis. A key aspect is external validation, both biochemical and imaging biomarkers require external validation to support their use

in clinical practice. This step is the most dangerous and where many biomarkers lose interest and applicability. A great consortium with academic and industry centers working together on all stages of this disease could be the better scenario to develop these biomarkers we need in clinical practice to improve continuous of care of patients with NAFLD.

Contents

Chapter 1
The Spectrum of NAFLD: From the Organ to the System

Yolanda Sanchez-Torrijos and Javier Ampuero

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinical-pathological condition that encompasses a wide range of liver damage not caused by chronic alcohol consumption, including steatosis, non-alcoholic steatohepatitis (NASH) and cirrhosis [1], in the absence of other etiologies. It is associated with metabolic risk factors such as obesity, dyslipidemia, and diabetes mellitus [2, 3]. The prevalence of NAFLD has increased considerably over the last years due to the current lifestyle (unhealthy diet and sedentarism) [4, 5]. It has been calculated that up to 30% of the population shows NAFLD, representing up to 70% in patients with type 2 diabetes mellitus [6]. Despite the high prevalence of NAFLD, the vast majority of patients show simple steatosis, which is a benign condition, while a small percentage of them have NASH or liver fibrosis [7].

Although NAFLD is considered the hepatic manifestation of the metabolic syndrome, there is an increasing number of studies that support a bidirectional model, in which NAFLD plays a crucial role in the development of metabolic disturbances [8]. Also, there is strong evidence that supports the multisystemic affectation of NAFLD, including cardiovascular, bone and kidney disease, and promoting hepatic and extrahepatic malignancies [9].

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NAFLD: Spectrum of Disease

The diagnosis of NAFLD is based on the presence of steatosis in ≥5% of hepatocytes showing by histology [10], while NASH is characterized by the presence of steatosis, hepatocellular damage (in the form of ballooning degeneration, apoptosis or necrosis) and lobular inflammatory infiltration. The presence of mild fibrosis is common in NASH, but it is not a requirement for the histological diagnosis [11]. The determination of NASH provides important prognostic information and indicates an increased risk of progression to fibrosis, cirrhosis, and some NAFLD-related comorbidities [12]. Fibrosis is the most important prognostic factor in NAFLD and correlates with liver outcomes and mortality [13]. NAFLD is now considered the second most common indication for liver transplantation in the USA after chronic hepatitis C, and it is still growing [14]. Therefore, it is essential to identify patients who present advanced fibrosis since they will require a detailed assessment and an adequate follow-up.

NAFLD and Cardiovascular Disease

The association between NAFLD and cardiovascular risk underlies on sharing some epidemiological (i.e., obesity, insulin resistance, diabetes mellitus, sedentary lifestyle, arterial hypertension, or dyslipidemia) and genetic risk factors (i.e., PNPLA3, TM6SF2) [15].

On the one hand, NAFLD increases the risk of subclinical atherosclerosis and myocardial dysfunction. Ampuero et al. [16] performed a meta-analysis including 14 studies, evaluating the presence of subclinical atherosclerosis (measured by the carotid artery intima-media thickness (CIMT) and the presence of carotid plaques) and coronary artery disease in patients with NAFLD. This latter was detected either abdominal ultrasound or liver biopsy. Individuals with NAFLD showed a higher risk of pathological CIMT (OR 2.04 (95% CI; 1.65–2.51)), presence of carotid plaques (OR 2.82 (95% CI; 1.87–4.27)) and coronary artery disease (OR 3.31 (95% CI; 2.21–4.95)). In addition, VanWagner et al. [17] assessed if patients with NAFLD underwent echocardiography with myocardial strain showed subclinical myocardial remodeling or dysfunction. They found that NAFLD was independently associated with subclinical myocardial remodeling and dysfunction, particularly they had lower early diastolic relaxation, higher left ventricular filling pressure, and more significant ventricular systolic dysfunction.

On the other hand, there is an increased risk for cardiovascular and cerebrovascular events in patients showing NAFLD compared with healthy controls. Fracanzani et al. [18] demonstrated that the presence of plaques (OR 5.08 (95% CI; 2.56–10.96)) and liver steatosis (OR 1.99 (95% CI; 1.01–3.94)) were the strongest predictors of cardiovascular outcomes during 10 years of follow-up. These findings have been supported by a recent meta-analysis that included 34 studies (164,494

participants, 21 cross-sectional studies, and 13 cohort studies), with an increased risk of prevalence disease (OR 1.81 (95% CI; 1.23–2.66) and incidence of cardiovascular (HR 1.37 (95% CI; 1.10–1.72)) [19].

Consequently, the cardiovascular risk is one of the leading causes of mortality in patients with NAFLD. This was demonstrated by Ekstedt et al. [13], who followed-up 229 patients during 33 years. They observed that cardiovascular disease was the main cause of mortality in patients with NASH and liver fibrosis. By contrast, the liver-related outcomes were the major prognostic determinants in patients with NAFLD-related cirrhosis.

Taking all together, the *European Association for the Study of the Liver* [20] recommends screening for cardiovascular disease in NAFLD patients, evaluating at least, for traditional risk factors including obesity, diabetes, dyslipidemia and hypertension. In addition, there are different methods easy to use in the general population to estimate 10-year risk of cardiovascular disease (i.e. the Framingham Risk Score that has been validated in NAFLD patients).

NAFLD and Kidney Disease

Given that NAFLD and CKD share the main risk factors, such as hypertension, obesity, dyslipidemia, and insulin resistance, it is difficult to determine the causal relationship between both entities. In addition, most of the studies associating NAFLD and CKD show small sample sizes and a non-definitive diagnosis of NAFLD, usually based on ultrasound instead of liver histology [21].

Interestingly, CKD may mutually aggravate NAFLD and associated metabolic disturbances through altered intestinal barrier function and microbiota composition, the accumulation of uremic toxic metabolites, and alterations in pre-receptor glucocorticoid metabolism [22].

Chronic kidney disease (CKD) can be seen in 20–50% of patients with NAFLD, according to several studies. One of the first studies assessing this relationship was performed by Target et al. [23], which included 80 overweight patients with biopsy-proven NASH and 80 non-steatotic control subjects. Authors concluded that NASH patients had CKD more frequently, independently of traditional risk factors. Muso et al. [24] carried out a large meta-analysis, including 33 studies, to evaluate the association between NAFLD (diagnosed by histology, imaging techniques or biomarkers) and CKD. Authors assessed the impact of NAFLD, NASH, and advanced fibrosis on the prevalence and incidence of CKD. First, NAFLD was associated with an increased risk of prevalent (OR 2.12 (95%CI; 1.69–2.66)) and incident (HR 1.79 (95%CI; 1.65–1.95)) CKD in comparison with healthy controls. Second, NASH was associated with a higher prevalence (OR 2.53 (95%CI; 1.58–4.05)) and incidence (HR 2.12 (95%CI; 1.42–3.17)) of CKD than simple steatosis. Third, advanced fibrosis was associated with a higher prevalence (OR 5.20 (95%CI; 3.14–8.61)) and incidence (HR 3.29 (95% CI; 2.30–4.71)) of CKD than non-advanced fibrosis. These results were not affected by other confounding factors such as diabetes mellitus.

Although the assessment of annual glomerular filtration in NAFLD patients should be considered [25], more prospective studies are needed to help adequately to make clear recommendations to the early detection and treatment of CKD.

NAFLD and Liver Tumors

Hepatocellular carcinoma (HCC) is the most frequent tumor in the liver and, irrespective of the underlying cause of the liver disease (i.e., alcohol, viral hepatitis), the presence of metabolic risk factors increases its prevalence. In fact, relevant elements in the pathogenesis of NAFLD, such as diabetes mellitus (insulin resistance) and obesity (adipose tissue deposit, and inflammatory cascade), are essential for the impairment of signaling pathways, which eventually lead to the development of HCC [26].

Obesity is associated with the occurrence of HCC. In a meta-analysis conducted by Larsson et al. [27], including observational studies, subjects with higher body mass index showed a greater risk of developing HCC. Besides, the presence of early adulthood obesity has been associated with an increased risk of developing HCC at a younger age in the absence of other major HCC risk factors [28]. In addition, obesity may exacerbate the impact of chronic hepatitis B or C infection on HCC, from 31.7 (95% CI; 19.3–52.3) in case of hepatitis B or C infection alone to 72.5 times (95% CI; 9.2–574.2) with the simultaneous presence of the virus infection and obesity [29]. Similarly, diabetes mellitus and metabolic syndrome impact on the development of HCC. Since the 1990s, several studies documented this association [30, 31], which was confirmed in a later meta-analysis performed by Tanaka et al. [32]. They found a relative risk of 2.18 (95% CI; 1.78–2.69) for the development of HCC in patients with diabetes mellitus.

NAFLD is currently accounting for 15% of all causes promoting HCC, showing an annual increase of 9% as risk factor [33, 34]. Interestingly, the profile of NAFLD patients changes compared to other etiologies, because they are older, show higher metabolic-associated comorbidity, and up to one-third of them may be non-cirrhotic [35]. NAFLD-related HCC has been found to be often detected at a later tumor stage (a larger volume and a more often infiltrative pattern) than HCV-associated HCC. Regardless of tumor stage, survival was significantly shorter in patients with NAFLD-HCC (25.5 months) than in those with HCV-HCC (33.7 months), probably because the first group of patients was more often out of the Milan criteria. However, the survival rate was similar between patients within Milan criteria [36]. Interestingly, cirrhosis was present in only about 50% of NAFLD-HCC patients, in contrast to the near totality of HCV-HCC. Despite, there is no current recommendation to screen HCC in NAFLD patients with no cirrhosis [37].

NAFLD and Extrahepatic Malignancies

In addition to HCC, overweight or obesity have shown to increase the risk of cancers related to endometrium, kidney, gallbladder (mainly, in women), breast (in postmenopausal women), colon (particularly, in men) and adenocarcinoma of the esophagus [38]. In a large US study, that included a total of 900,053 participants, the subjects with morbid obesity had death rates associated with all cancers combined 52 and 62% higher than the rates in men and women of normal weight, respectively. In both men and women, BMI was significantly associated with higher rates of death due to cancer of the esophagus, colon and rectum, liver, gallbladder, pancreas, and kidney, as well as for death due to non-Hodgkin's lymphoma and multiple myeloma [39].

In one of the largest studies published to date, Kim et al. [40] aimed to evaluate the incidence of extrahepatic cancer during a follow-up period of 7.5 years. They found an increase in the incidence of any type of cancer in NAFLD patients, with a cancer incidence rate of 782.9 vs. 592.8 per 100,000 person-years (HR 1.32 (95% CI; 1.17–1.49)). Notably, NAFLD showed a strong association with colorectal cancer in males (HR 2.01 (95% CI; 1.10–3.68)), and breast cancer in females (HR 1.92 (95% CI; 1.15–3.20)). Interestingly, the incidence of tumors in NAFLD was associated with a higher fibrosis stage.

Colorectal cancer has been probably the extrahepatic tumor more associated with NAFLD. The activation of the inflammatory cascade and the increase in the activity of the pathways involved in cell proliferation, which are typically exacerbated in NAFLD, play an essential role on the development of colorectal cancer in these patients [41]. In 2012, Lee et al. [42] observed an adjusted twofold increase in the occurrence of adenomatous polyps and a threefold increase in colorectal cancer in patients with NAFLD compared with healthy individuals. In 2017, Ahn et al. [43] analyzed the risk of colorectal neoplasia according to the presence of NAFLD. They included 26,540 asymptomatic adults who underwent the same day first-time colonoscopy and abdominal ultrasonography as a health check-up programme. The presence of NAFLD showed a higher prevalence of any colorectal neoplasia (38.0% vs. 28.9%) and advanced colorectal neoplasia (2.8% vs. 1.9%) compared to the absence of fatty liver. In addition, the risk of colorectal neoplasia was higher in NAFLD patients with greater fibrosis stage, according to non-invasive tests.

More studies are needed to accurately assess the relationship between NAFLD and the different types of cancer and evaluate the usefulness of screening programs focused on these patients.

NAFLD and Bone Disease

Osteoporosis can be aggravated by the presence of insulin resistance [44], which typically occurs in conditions leading to NAFLD, such as central obesity, diabetes, and metabolic syndrome [45]. In addition, NAFLD-related systemic inflammation (inflammatory cytokines, especially TNF-a, IL-1, IL-6, and osteopontin) have been implicated in osteoporosis [46]. On the other hand, the liver is also the source of many proteins involved in bone metabolism, as well as it is a regulator of several bone metabolism pathways.

NAFLD has been associated with osteoporosis in cross-sectional and longitudinal studies. First, Monn et al. [47] included 480 pre and post-menopausal women to assess the impact of fatty liver (measured by ultrasound) on the presence of low bone mass. They observed that the bone mass was lower in subjects with NAFLD than those without it in postmenopausal (0.98 ± 0.01 vs. 1.01 ± 0.02 g/cm^2), but not in premenopausal women. This relationship was independent of other factors such as age, BMI, smoking status, and alcohol consumption. Second, Chen et al. [46] conducted a retrospective study (mean follow-up ten years) in a Taiwanese cohort, including patients with (n = 4318) and without (n = 17,272) NAFLD. They found that patients with NAFLD were 1.35 times more likely to develop osteoporosis compared with those without NAFLD (95% CI; 1.20–1.53). Authors finally raised awareness about the early detection of osteoporosis in NAFLD patients. These results have been similar in adolescents, where NAFLD was essential for obese children to be more susceptible to osteoporosis [48]. However, a recently published meta-analysis of observational studies found no significant difference in bone mineral density between patients with fatty liver disease and controls [49]. Therefore, and given the current controversy regarding the association of both disorders, more studies are needed to make final recommendations about the evaluation of osteoporosis in NAFLD patients.

NAFLD and Metabolic Disturbances

Most of the patients with NAFLD have, at least, one of the characteristics of the metabolic syndrome, and up to a third of them have a complete diagnosis [50]. However, there is currently convincing evidence to suggest that NAFLD may often precede the development of type 2 diabetes mellitus or other components of the metabolic syndrome [51]. In patients with NAFLD, both genetic and environmental factors (through the lipotoxic effect) can interfere with the insulin signaling pathway contributing to maintain or worsen the insulin resistance.

The effect of NAFLD on diabetes mellitus has been extensively evaluated. In a prospective cohort, enrolling 25,232 Korean men without type 2 diabetes mellitus and followed-up for 5 years, the incidence rate of diabetes increased according to the steatosis degree (normal: 7.0%, mild: 9.8%, moderate to severe: 17.8%, $p < 0.001$).

Even after adjusting for other well-documented risk factors, the incidence of diabetes was higher in the mild (HR 1.09 (95% CI; 0.81–1.48)) and moderate to severe groups (HR 1.73 (95% CI; 1.00–3.01)) compared to the normal subset, respectively [52]. Yamazaki et al. [53] observed similar results (16.1% vs. 3.1% for the incidence of diabetes in NAFLD and non-NAFLD patients, respectively) but, interestingly, they found that the improvement of NAFLD during the follow-up was associated with a reduction of more than 70% in the risk of developing diabetes. Björkström et al. [54] included 396 patients, who did not have type 2 diabetes at baseline, diagnosed with NAFLD by liver biopsy. They found that the development of diabetes was significantly greater at a higher fibrosis stage (advanced fibrosis = 51.2% vs. mild fibrosis = 31.3%). Besides, fat score was associated independently with the development of type 2 diabetes in lower stages of fibrosis (HR 1.34 (95% CI; 1.03–1.74).

The *European Association for the Study of the Liver* recommends the annual evaluation of HbA1c to screen diabetes mellitus in patients with NAFLD [20].

Conclusions

The clinical burden of NAFLD extends beyond liver-associated mortality since it impacts on a lot of extrahepatic organs and interacts with the regulation of multiple metabolic pathways. NAFLD increases the risk of occurrence relevant extrahepatic entities, such as cardiovascular and kidney disease, as well as recent data indicate an additional risk for some tumors and osteoporosis. Thus, clinicians should raise the awareness of these increased extrahepatic risks in NAFLD patients. However, there is a lack of studies evaluating a cause-and-effect relationship properly, so future researches must be warranted to decipher the link between NAFLD and the development and progression of extrahepatic chronic diseases.

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Chapter 2
Detection of NAFLD/NASH in the General Population and in Primary Care Clinics

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is emerging as the most common chronic liver disorder, affecting approximately 25% of the population globally. The association of NAFLD with metabolic morbidity and cardiovascular disease (CVD), as well as its association with significant liver-related morbidity and mortality, create important challenges for primary care physicians in relation to the prevention, diagnosis and treatment of NAFLD and its associated risks for CVD and liver-related morbidity [1]. The importance of establishing a policy in regards to early detection of NAFLD and advanced fibrosis in primary care settings or diabetes clinics, among people with obesity, diabetes or the metabolic syndrome, who represent high-risk populations for the more advanced forms of NAFLD, is increasingly recognized. As most NAFLD patients in primary care settings have simple steatosis (NAFL), and are not at increased risk for liver-related morbidity, it is extremely important to provide physicians who see NAFLD patients in primary care settings and diabetes clinics with tools to identify patients at high liver-related risk, who will benefit from specialist care. The implementation of predictive models for risk stratification may change the landscape of early detection in non-specialist clinical settings. From a

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public health perspective, primary prevention policies and actions may be implemented for subjects categorized as being at high risk according to these models. Some of the tools incur minimal additional costs, being based on readily available lab tests, and can be calculated automatically in computerized medical systems. Their availability can be harnessed as a tool to increase awareness among general practitioners, diabetologists and the public to promote early diagnosis and appropriate management. Although, as for now, there are no approved drugs for NASH, and treatment focuses mainly on lifestyle modification, awareness may increase motivation among patients to improve their lifestyle, and intensify monitoring for liver-related (e.g. occult cirrhosis, hepatocellular carcinoma) and cardiovascular risks by physicians following the patient. With the advent of pharmaceutical treatments for NASH, which are expected in the near future, low cost, readily available prediction models will assist in identifying suitable patients for treatment.

The Epidemiology of NAFLD

NAFLD encompasses two pathologically distinct conditions with different prognoses: non-alcoholic fatty liver (NAFL) which is pure steatosis with or without mild lobular inflammation, and non-alcoholic steatohepatitis (NASH), that can progress to liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC). Approximately 20% of NAFL patients progress to NASH with an average progression rate of 11% during a 15-year period; the fibrosis progression rate is highly variable and influenced, to a large extent, by metabolic risk factors. A meta-analytic assessment of the global epidemiology of NAFLD estimates that 40% of NASH patients progress to fibrosis with an annual fibrosis progression rate of 0.09%. The proportion of NASH among the NAFLD population is estimated to increase in the coming decades due to an aging population and the rising prevalence of type-2 diabetes mellitus (T2DM) and obesity [1–3]. NASH has been recognized as one of the leading causes of cirrhosis in adults in the United States, and NASH-related cirrhosis is currently the second indication for liver transplants in the United States [4].

Within the NAFLD spectrum, the prognosis of NAFL differs significantly from that of NASH, with no increase in liver-related mortality and minimal risk for disease progression. However, NASH with fibrosis progresses at a faster rate than NASH without fibrosis, with high risk of developing HCC, liver failure, and death [5, 6]. Although evidence clearly supports the development of HCC in patients with NASH-cirrhosis, data now suggest that HCC can also occur in NASH patients without advanced fibrosis. It is estimated that the yearly incidence of HCC among NASH-cirrhotic patients is 2–3% and that the annual incidence of HCC in NAFLD patients is 0.44 per 1000 person-years [1, 2].

The prevalence of NAFLD is increasing worldwide. A meta-analytic assessment of the global epidemiology of NAFLD estimates that the global prevalence of NAFLD is ~25% with the highest prevalence in the Middle East and South America, and the lowest prevalence in Africa. It is the most common liver disease in Western

countries, affecting 17–46% of adults, with differences in prevalence values stemming from variability related to diagnostic method, age, sex and ethnicity [7].

The prevalence of NAFLD is influenced by the diagnostic modality. Use of elevated liver enzymes as the primary diagnostic tool significantly underestimates the prevalence of NAFLD compared to abdominal ultrasonography (AUS) and liver biopsy. It has been shown that aminotransferase levels may be only mildly elevated in NAFLD, and that more than 50% of NAFLD patients may have normal liver enzymes. In a prospective cohort study, in which NAFLD diagnosis was based on ultrasound and liver biopsy, the prevalence of NAFLD and NASH in asymptomatic middle-aged patients in the United States was found to be 46% and 12.2%, respectively, compared to 13% for an elevated aminotransferase-based NAFLD diagnosis [8].

The age and gender distributions of NAFLD vary. It is assumed that the overall prevalence of NAFLD is approximately 30–40% in men and 15–20% in women, with prevalence rates increasing with age and the presence of T2DM [9]. Furthermore in regards to age, Kohler et al. found that advancing age is associated with clinically relevant liver fibrosis in patients with NASH, in the presence or absence of T2DM [10].

An ethnic variation in the distribution of NAFLD has also been suggested; in the US, Hispanics have the highest prevalence (45–58%), followed by whites (33–44%) and blacks (24–35%). These variations are probably secondary to lifestyle and genetic predisposition, as Hispanics with NASH tend to be younger, less active, and with unhealthy dietary habits [2, 11]. There are distinct phenotypes of NAFLD in different regions of the world, secondary to complex interactions between genetics, diet, the microbiome, and other environmental influences on the development of NAFLD. Previous studies showed that patients with NAFLD from East Asia have lower BMI and higher T2DM rates than patients in the West. This "Asian paradox" may be secondary to distinct genetic or environmental susceptibility to NAFLD, that differs from that of individuals in the West [12].

Obesity, T2DM and the metabolic syndrome are consistently identified as the most important risk factors for NAFLD, fibrosis and CVD. The prevalence of NAFLD in patients with T2DM is 40–70%. Kohler et al. have found a strong association between increased liver stiffness and presence of T2DM and/or insulin resistance, suggesting that having T2DM, especially in the presence of NASH, may result in an increased risk of clinically relevant fibrosis, cirrhosis, and mortality [10].

The prevalence of NAFLD in obese or morbidly obese patients is 75–92%, and over 90% of morbidly obese patients undergoing bariatric surgery have NAFLD [13]. According to a meta-analytic assessment of the global epidemiology of NAFLD, the pooled overall obesity prevalence among NAFLD and NASH patients is ~ 50% and ~80% respectively, with the highest rates in North America (86%) [1].

NAFLD has been referred to as the hepatic manifestation of the metabolic syndrome. It is increasingly viewed as an independent contributor to cardiovascular risk, via insulin resistance, oxidative stress, worsening inflammatory state and endothelial dysfunction [1], that accelerate the development and progression of atherosclerosis and arterial stiffness. Multiple epidemiological studies have linked

NAFLD to increased CVD, concluding that, although the primary liver pathology in NAFLD involves morbidity and mortality from cirrhosis, liver failure and hepatocellular carcinoma, the majority of deaths among NAFLD patients are attributable to CVD [9]. In fact, death from cardiovascular causes in patients with NAFLD is twofolds higher than death related to liver disease [14].

To summarize, ~25% of the adults in the developed world have NAFLD, with a large proportion of these patients having T2DM and obesity, that increase the risk for progression to NASH, cirrhosis and HCC, and liver-related mortality. Alongside the liver-related risk, NAFLD is increasingly recognized as an independent risk factor for cardiovascular disease, and most patients with NAFLD die from CVD. The increasing prevalence of T2DM and obesity, in conjunction with aging of the population, calls for screening and early treatment strategies to prevent potentially life-threatening hepatic and cardiovascular complications.

The Approach to Diagnosing NAFLD in Primary Care Settings and Risk Stratification

In primary care settings, patients with NAFLD are usually diagnosed for one or more of the following reasons: (1) in the framework of an investigation for elevation of liver enzymes (2) due to evidence of a fatty liver in AUS undertaken for another reason (3) as part of case finding. Patients in the last two categories may not have elevated liver enzymes.

Clinical assessment involves three practical steps:

The first step is to identify patients who should be screened for NAFLD; the second step is to diagnose NAFLD, and to rule out etiologies other than NAFLD for liver fat accumulation or elevated liver enzymes; the third step, risk stratification, involves identification of patients who are at risk of fibrosis and liver related outcomes, as advanced liver fibrosis is associated with increased overall mortality and liver related events [15], and referral of these higher-risk patients to specialist care in hepatology clinics.

Step 1: Who Should Be Screened for NAFLD?

There is no universal directive for systematic screening of the general population for NAFLD for the following reasons: (1) Although NAFLD is a common disease, the prevalence of severe complications in the general population is low; (2) There are currently no approved drug therapies. (3) Lack of large scale cost effectiveness analysis. Compared to the low prevalence of severe complications in the general population, the prevalence/incidence of fibrosis and HCC increases in diabetic and obese persons. Thus, there is a consensus among some of the international professional associations to screen obese and T2DM patients for NAFLD [16–19]. The European

Table 2.1 Screening recommendations for NAFLD

Professional association	EASL [16]	AASLD [20]	NICE [17]	AISF [21]
Systematic screening	No	No	No	No
Screening in high risk populations	Diabetes and/or metabolic risk factors; obesity, metabolic syndrome, persistently abnormal liver enzymes	No	Obesity, diabetes	No
Modalities of screening	liver enzymes Abdominal US		Abdominal US	

Adopted from [22]

Association for the Study of the Liver (EASL) also recommends screening patients with features of the metabolic syndrome by liver enzymes and or AUS. It is emphasized that the presence of NAFLD should be assessed in these patients irrespective of the liver enzymes level, since T2DM patients are at high risk of disease progression [16]. In contrast, the American Association for the Study of Liver Diseases (AASLD) and the Italian Association for the Study of the Liver (AISF) do not recommend screening for fatty liver [20, 21]. Nonetheless, the AASLD recommends a high index of suspicion for NAFLD and NASH in patients with T2DM (Table 2.1).

Step 2: Diagnosing NAFLD

NAFLD is usually detected either by investigation of abnormal liver enzymes or following incidental detection of hepatic steatosis on AUS. Detection of these patients may also occur in the framework of screening programs for NAFLD in high risk patients. In all cases, it is important to rule out other liver diseases, including alcoholic liver disease (ALD), viral hepatitis, drug related, autoimmune or metabolic disease, that may cause steatosis or elevation of liver enzymes.

Standard Blood Tests

Elevated liver enzymes are the most common blood test abnormality to trigger an investigation for NAFLD, but have important drawbacks. It is estimated that liver enzymes may be normal in up to 80% of NAFLD patients [11, 23]. When liver enzymes are elevated, the aberration is commonly a slight to modest elevation of alanine transaminase (ALT) and aspartate transaminase (AST). AST and ALT levels are usually between ×2–5 the upper limit of normal (levels above 300 IU/L are rare), with an AST:ALT ratio <1 [24]. An AST: ALT ratio >2 increases the likelihood of alcoholic liver disease, and this likelihood increases further if the ratio exceeds 3. Gamma glutamyl transferase (GGT) may represent a complementary test to identify patterns of alcoholism or alcohol abuse, but GGT by itself is not helpful in estab-

lishing a diagnosis of alcoholic liver disease [24]. Elevated carbohydrate deficient transferrin (CDT) and high MCV may also imply chronic alcohol consumption. The sensitivity for detection of daily ethanol consumption of more than 50 g is 69% for CDT and 73% for GGT. The specificity is 92% for CDT and 75% for GGT, respectively [25]. An AST/ALT ratio >1 is also characteristic of cirrhosis (of any etiology) [24].

Although many individuals with NAFLD, suggested by hepatic steatosis on imaging, may have normal liver enzymes, the presence of abnormal liver enzymes signals a higher likelihood for NASH with or without fibrosis, and warrants further clinical evaluation [24]. It is important to note that the degree of aminotransferase elevation does not predict the extent of hepatic injury, and having normal liver enzymes is not synonymous to the absence of steatosis or fibrosis in adults as well as in pediatric NAFLD [23, 26–28]. On the other hand, NASH resolution following weight reduction by lifestyle intervention was demonstrated to be strongly related to normalization of ALT (≤19 in females or ≤30 in males) [29]; leading to the development of a NASH resolution calculator (http://www.aeeh.es/calculadora-nashres/), in which normalization of ALT is an item [30].

Another marker of liver damage is serum ferritin. Elevation of serum ferritin levels is common in NAFLD patients, and usually indicates disease progression. There is evidence that serum ferritin greater than 1.5 times the upper normal limit is associated with the diagnosis of NASH and advanced hepatic fibrosis in both males and females [31–34]. Notably, in patients with high serum ferritin and increased iron saturation, the AASLD recommends exclusion of hemochromatosis [20].

Diagnostic Modalities for Steatosis and NASH

A number of diagnostic radiological imaging modalities can confirm the presence of hepatic steatosis—AUS, CT, MRI, and FibroScan Controlled Attenuation Parameter (CAP). Use of AUS is the most commonly used first-line imaging modality to assess for suspected NAFLD. Its main advantages are low cost and broad availability, but it has limited sensitivity in morbidly obese patients, and in the presence of less than 20% steatosis (assessed by liver biopsy) [16, 20].

Some serum markers can also detect steatosis, but with limited validity. Such markers are usually used for large scale screening studies and not in the setting of primary care clinics. Better validated steatosis scores include the Fatty Liver Index (FLI) [35], the lipid accumulation product (LAP) [36] and the Steatotest [37] (costly and not available in clinical practice). The FLI takes into account BMI, waist circumference, triglycerides and GGT levels (a free web-based calculator is available). A score ≤ 30 has a sensitivity of 87%; and a score ≥ 60 has a specificity of 86% for detection of hepatic steatosis [35].

Steatohepatitis is a histological diagnosis, defined as the combined presence of steatosis (>5% of hepatocytes), inflammation and hepatocyte injury (ballooning). At present, there are no well-established biomarkers to distinguish NASH from

NAFL. Circulating levels of keratin 18 fragments (CK-18), which are released from apoptotic or dead cells, have been extensively investigated. However, CK18 has limited validity as a screening test for NASH and is currently not available in clinical care settings [38, 39].

Step 3: Risk Stratification of NAFLD Patients:

Liver fibrosis is the only parameter that was found to be correlated with liver-related morbidity, liver transplantation and liver-related mortality in patients with NAFLD. Therefore, risk stratification, based on the presence or absence of advanced fibrosis, is recommended in this patient population [40–42]. Patients with advanced fibrosis or cirrhosis (Metavir stages F3 or F4, respectively) are at risk for clinically significant liver outcomes (i.e. complications of cirrhosis, need for liver transplantation or liver-related death), and should be referred to specialist care for early detection and management of cirrhosis and its complications. Patients with no fibrosis or minimal fibrosis (Metavir stages F0 or F1, respectively) are considered to be at low risk for liver-related outcomes, and can be followed in primary care settings, with periodic reassessments [16, 42].

A number of clinical factors may suggest an increased risk for advanced liver fibrosis, including age ≥50, male gender, alcohol consumption, severe obesity, and presence of the metabolic syndrome (the risk for advanced fibrosis increases with increasing metabolic burden), elevated transaminases (≥×2 upper limit of normal), and an elevated ferritin level [20, 31, 43–47]. T2DM is also associated with more severe manifestations of NAFLD, including advanced fibrosis, cirrhosis and HCC [48]. Thus, the pre-test probability of advanced fibrosis in an obese 55 year-old patient with T2DM and other features of the metabolic syndrome is significantly higher than that in a young, overweight patient without these comorbidities. This should be taken into account in risk assessment, as NAFLD patients with multiple clinical risk factors may benefit from early referral to specialist care [49]. Finally, patients in whom there is suspicion for cirrhosis (e.g. compatible physical examination findings, AST/ALT >1 in the absence of alcohol consumption, splenomegaly, thrombocytopenia) should be referred promptly for further evaluation and management in a liver clinic.

While liver biopsy remains the gold standard for diagnosing NASH and fibrosis in patients with NAFLD [20], it is not feasible or justified in all NAFLD patients. Consequently, a number of non-invasive measures have been developed, that aid in classification of patients with NAFLD into those who are, or are not, at increased risk for advanced fibrosis [50–54]. The use of non-invasive tools to assess liver fibrosis for initial risk stratification in clinical settings is endorsed by professional societies [16] and is becoming widespread, while liver biopsies are increasingly reserved for situations in which: (a) a diagnostic question remains as to whether the patient has NAFLD or another liver disorder—for example, in patients with

significant liver enzyme elevations or high titres of autoimmune antibodies, patients without metabolic syndrome, etc. Notably, given the high prevalence of NAFLD, it is not uncommon for patients with other liver disorders to also have NAFLD. (b) To accurately establish or confirm the degree of histological damage to the liver, particularly in subjects who are suspected to have advanced fibrosis or cirrhosis based on high-risk clinical features [49], or suggestion of advanced fibrosis by non-invasive tests.

Currently available, commonly used non-invasive tools to classify NAFLD patients into those who are at high versus low risk for advanced fibrosis include laboratory test-based risk scores (e.g. Fibrosis 4 (FIB4); NAFLD fibrosis score (NFS) or the Enhanced Liver Fibrosis (ELF) panel), and imaging modalities (e.g. Fibroscan—Vibration Controlled Transient Elastography (VCTE)—the most widespread and studied of these methods; Acoustic Radiation Force Impulse elastography, and Magnetic resonance elastography (MRE)). It is notable that all of these methods have been validated in comparison to liver biopsy as a gold standard, which has imperfect accuracy by itself (due to sampling error and intra- and inter-observer reliability), leading to an inherent bias in the performance accuracy of these non-invasive tests.

Non-invasive Assessment of Fibrosis

Laboratory Test-Based Risk Scores

A number of risk scores have been developed based on readily available clinical and laboratory parameters that are simple to use at point of care with the help of web-based calculators, and can be implemented into computerized medical systems. These include, among others, the APRI (AST to platelet ratio index) [55], BARD (BMI; AST/ALT ratio; diabetes) score [56], FIB4 (Age; AST; ALT; platelets) and NFS (Age; BMI; AST; ALT; albumin; impaired fasting glucose/diabetes) scores. Of these, FIB4 and NFS have been most extensively studied and validated in diverse populations, and shown to predict overall mortality, cardiovascular mortality and liver-related mortality in patients with NAFLD [16]. FIB4 and the NFS are currently recommended for the initial assessment of subjects with NAFLD and metabolic risk factors [16, 20, 42], who are older than 35 years of age (alternative modalities for fibrosis assessment are recommended in younger patients) [57]. FIB4, which is calculated as Age × AST (IU/L)/platelet count (×109/L) × $\sqrt{}$ALT (IU/L), has an area under the receiver operating characteristic curve (AUROC) >0.8 for detection of stage F3 or F4 fibrosis. The NFS is calculated as: $-1.675 + 0.037 \times$ age (years) $+ 0.094 \times$ BMI (kg/m^2) $+ 1.13 \times$ impaired fasting glycemia (IFG) or diabetes (yes = 1, no = 0) $+ 0.99 \times$ AST/ALT ratio $- 0.013 \times$ platelet (×10^9/l) $- 0.66 \times$ albumin (g/dL); an AUROC 0.85 for detection of advanced fibrosis was reported for the NFS in a meta-analysis of 13 studies, that included 3064 patients with NAFLD [14]. Commercial laboratory test-based risk scores for fibrosis include such tests as the proprietary ELF® panel, Fibrotest®, FibroMeter® and Hepascore® [52, 58].

In contrast to the FIB4 and NFS, that incur minimal additional costs, being based on routine lab tests that have often already been done in the patient, these tests are proprietary algorithms that include non-routinely tested parameters (e.g. for Fibrotest®: alpha 2 macroglobulin, haptoglobin and apolipoprotein A1 [59]; for ELF®: procollagen III amino terminal peptide, hyaluronic acid and tissue inhibitor of matrix metalloproteinase-1, which are direct markers of fibrosis [60, 61]), carry additional costs, and depend on local availability. For Fibrotest®, which has been validated in NAFLD as well as other common liver diseases, a mean standardized AUROC for advanced fibrosis of 0.84 (95% CI 0.76–0.92) has been reported. The AUROC, sensitivity and specificity of the ELF panel have been reported to be 0.90, 0.80 and 0.90, respectively, for identifying advanced fibrosis [62]. The test has been approved for commercial use in Europe, and has been recommended as a test of choice for ruling out advanced fibrosis in NAFLD patients in a 2016 National Institute for Health and Care Excellence (NICE) guideline [17].

For the FIB4 and the NAFLD fibrosis score, two cut-off values are defined. Patients who score lower than the lower cut-off value (FIB4 score ≤ 1.3; NFS ≤ -1.455) [42, 57] can be regarded as having a low risk for advanced fibrosis, and do not need to be referred to a hepatology clinic at that point in time (in NAFLD patients $\geq$65 years of age, recommended lower risk cut-offs are <2.0 and <0.12 for the FIB4 and NAFLD fibrosis scores, respectively [57]). According to the current EASL recommendations, such patients should be non-invasively re-assessed after 2 years. In patients whose score is higher than the higher cut-off value (FIB4 score > 2.67; NFS >0.675), there is suspicion of advanced fibrosis or cirrhosis, and these patients should be referred to a hepatology clinic for further assessment. Thirty to fifty percent of patients have an indeterminate score, and in these cases, additional testing is needed (e.g. by the ELF panel or VCTE). Although 2 cut-off values have also been defined for the ELF panel, the NICE guideline refers to a single cut-off value (10.51) [In recently published guidelines from the British Society of Gastroenterology, the recommended cut-off is 9.5 [42].] for prediction of advanced fibrosis. According to the guideline, adult subjects with an ELF panel score below this cut-off can be reassured that they do not have advanced fibrosis, and should be followed up by an additional ELF test after 3 years [17].

Generalizability to Primary Care Settings

Most of the clinical and laboratory parameter-based risk scores have been developed and validated in specific patient populations attending liver clinics; this should be kept in mind when considering their widespread use in different patient populations (e.g. patients with diabetes, elderly patients, patients attending primary care clinics [63]). In line with this, questions have been raised regarding the generalizability of current cut-offs for all NAFLD patients. For example, it has been shown that the performance of FIB4 and NFS may differ with age [57], that the performance of APRI, BARD, FIB4 and NFS may differ with the degree of steatosis [64], that the performance of ELF may differ with age [65, 66] or gender [65] and that biomarker

panels for the diagnosis of NAFLD, NASH and advanced fibrosis (SteatoTest, ActiTest, NashTest and FibroTest) may underperform in patients with T2DM [67]. As the prevalence of NAFLD among patients with T2DM is high, and diabetes has been repeatedly shown to be a key predictor for advanced fibrosis, it is very important that non-invasive risk scores would be applicable to this patient population [10, 68, 69]. It was recently suggested that the frequency of indeterminate or high scores of fibrosis is higher in patients with T2DM. In a cross-sectional analysis of a study involving higher-risk patients with obesity, metabolic syndrome or diabetes, more than 84% of patients had indeterminate or high NFS or FIB4 scores, requiring further assessment [66]. A clinical model based on routinely available clinical and metabolic biochemical factors has been developed specifically for patients with T2DM, to determine the likelihood of NASH (AUROC 0.8) and advanced fibrosis (AUROC 0.8). The sensitivity and specificity for both NASH and advanced fibrosis was 57% and 90%, respectively. However, the main limitation of this tool was high percent of gray zone; 44% of patients could not be classified for NASH and 87% could not be classified for advanced fibrosis [70].

Liver Elastography-Based Assessment

Several liver elastography devices have been evaluated in cohorts of patients with NAFLD: vibration-controlled transient elastography (VCTE), shear-wave elastography (SWE), acoustic radiation force impulse (ARFI) imaging and magnetic resonance elastography (MRE). Fibrosis assessment is expressed as liver stiffness measure (LSM), measured in kilo-pascals (KPa); notably, the LSM ranges and cut-offs are different for the different modalities, and cannot be directly compared. Choice depends largely on availability and cost considerations. Advantages of SWE and ARFI include the combination of conventional ultrasound with liver stiffness measurements, allowing focus on an anatomic region of interest [71], and the capability to obtain liver stiffness values in patients with ascites [72, 73]. MRE may be more accurate than ultrasound-based modalities, has a lower risk of failure in patients with severe obesity, and measures a larger area of the liver, which may reduce sampling variability secondary to heterogeneity of fibrosis [74]. In a cross-sectional study of more than 100 patients, MRE was found to be more accurate than VCTE in identification of liver fibrosis (stage 1 or more), using biopsy analysis as the standard [75]. However, at present, MRE is expensive and not widely available in most geographies, and is used mostly in the setting of clinical trials. Currently, VCTE is the most common and widely clinically available diagnostic modality [76–79]. VCTE is performed using a Fibroscan® device (Echosens. Paris, France). It has been validated and found to be accurate across a wide spectrum of chronic liver disorders, and has important advantages, including: (1) It can be done at the point of care; (2) It is simple to learn; (3) It is well tolerated by patients; (4) Exam duration is short; (5) It assesses a liver volume that is ×100–200 greater than that assessed by a liver biopsy; and (6) There are standardized quality criteria [an adequate VCTE examination includes ten valid shots (>60% success rate), with an

interquartile range (IQR)-to-median LSM ratio of ≤0.3). In a meta-analysis of nine studies on VCTE, that included 1047 NAFLD patients, accuracy was moderate for F ≥ 2 (sensitivity and specificity 79% and 75%, respectively), and very good for F3 and F4 [sensitivity and specificity both 85% for F3; sensitivity and specificity both 92% for F4 [79]; AUROC 0.83 the prediction of F3/F4 fibrosis] [53]. Another advantage of VCTE is a simultaneous measurement of the controlled attenuation parameter (CAP) score that provides a quantitative assessment of hepatic steatosis [77]. Limitations of VCTE include high failure rates in patients with a narrow intercostal space or ascites, interference of liver stiffness measurements by extrahepatic cholestasis, elevated central venous pressure, post-prandial hepatic hyperaemia (patients should fast for 2–3 h) or acute liver injury, and reduced reproducibility in early stages of fibrosis and in the presence of steatosis [75, 80–82]. Notably, a relatively high failure rate that was reported for this modality in the past, especially in obese patients, has largely been overcome with the introduction of an additional probe (the XL probe), for use in subjects with BMI ≥30 kg/m^2 [76].

Approach to the Use of Non-invasive Fibrosis Assessment in Patients with NAFLD in Primary Care Settings

Recent guidelines recommend that calculation of FIB4 or NFS be the first step in risk assessment of NAFLD patients, to be done in primary care settings in which subjects with NAFLD are routinely followed. In fact, recent British Society for Gastroenterology guidelines recommend incorporation of calculation of FIB4 and NFS in all primary care computer systems [42]. Of interest in this regard, in healthcare records for 17.7 million adults from four large European primary-health-care databases, in which the FIB-4 could be calculated in 40.6% of patients, 1/3 had intermediate or high-risk scores [83]. According to current EASL guidelines, for subjects without liver enzyme elevation and with FIB4 or NFS scores consistent with a low risk for advanced fibrosis, follow-up should be by repetition of liver enzyme tests and FIB4 or NFS scores after 2 years [16]. Recent guidelines from the UK recommend repeat assessment after 2–5 years, depending on clinical risk [42]. Subjects whose liver enzymes are above the upper limit of normal or FIB4 or NFS scores above the higher cut-off should be referred to a hepatology clinic for further assessment. Subjects with indeterminate NFS or FIB4 scores can be referred to a second tier non-invasive assessment, such as VCTE or ELF; this approach is supported by studies that showed that combinations of non-invasive tests increase accuracy of prediction [16, 42, 74, 84, 85].

In subjects in whom the second non-invasive test indicates a low risk for advanced fibrosis, recommended follow-up is similar to that in subjects who were assessed as having a low risk for advanced fibrosis in the initial non-invasive test (FIB4 or NFS). The approach to NAFLD patients in whom a non-invasive test reveals advanced fibrosis is individualized, and adjusted to the subject's clinical features. When the initial test indicating a risk for advanced fibrosis is the FIB4 or NFS score,

a second tier non-invasive test (e.g. VCTE) is sometimes done. However, in view of suboptimal specificity and positive predictive value (PPV) of all non-invasive modalities, and taking into account the significance of cirrhosis diagnosis for the individual patient, the impact of this diagnosis on the use of healthcare resources and additional information that can be obtained from a liver biopsy, a permissive approach to referring such patients to a liver biopsy is usually practiced [42, 85].

Future Perspectives

There is an unmet need for additional non-invasive tools, that is likely to increase in the foreseeable future, with the advent of new therapies for non-alcoholic steatohepatitis [86]. The target population for such interventions, as reflected by recent guidance documents from the US Food and Drug Administration (FDA) [87] and European Medicines Agency (EMA) [88], is of patients who are at risk of cirrhosis, defined histologically as NAFLD activity score (NAS) $\geq$4 and F $\geq$ 2. Current non-invasive measures are not useful for detection of this population, and research is ongoing to develop non-invasive tools that would enable identification of relevant patients without a liver biopsy. The NIS4 algorithm, that is being commercially developed by Genfit and is based on four parameters (Alpha 2 macroglobulin, miR-34a, YKL-40 and Hemoglobin A1c) [89], and the FS3 [90] algorithm, that is based on fibroscan assessment (CAP and LSM scores) combined with AST, hold promise to meet this end. Another important area of unmet need pertains to accurate non-invasive follow-up of fibrosis in NAFLD patients over time. Current guidelines recommend periodic re-assessment of liver fibrosis by the available non-invasive tools (FIB4, NFS [16, 20, 42] or ELF). Re-assessments may indicate progression of fibrosis, as suggested by a recent study in which APRI, FIB4 and NFS could detect progression to severe fibrosis with a C statistic of 0.82, 0.81 and 0.80 respectively [91]. Non-invasive tools are sought that can more reliably differentiate between fibrosis stages as a continuum; in addition to indicating disease progression, such tools may be useful to monitor the therapeutic benefit of new treatments for NASH.

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Chapter 3
Genes and NAFLD/NASH Progression

Rasha El Sharkawy, Jacob George, and Mohammed Eslam

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a reversible condition that results from excess fat deposition in the liver (hepatic steatosis), in the absence of significant alcohol consumption [1]. However, with accumulating recent evidence suggesting that NAFLD is a global epidemic that affects about 20–30% of the population in most developed and developing countries, and up to 34% of obese children [2]. While NAFLD is strongly associated with obesity and metabolic syndrome, a significant proportion of individuals develop the disease in the context of a normal body mass index (BMI), who are considered to have 'lean NAFLD', though they remain poorly characterized [2]. Patients with lean NAFLD experience similar complications and tend to have worse outcomes compared to those with NAFLD that arises in the context of overweight and obesity [3, 4].

The spectrum of disease progresses through highly dynamic histological stages that includes simple steatosis at one end and an inflammatory subtype at the other, in which the presence of inflammation and ballooning signifies the presence of non-alcoholic steatohepatitis (NASH). The latter can progress to fibrosis, sometimes resulting in the development of cirrhosis, to liver failure, and/or cancer (hepatocellular carcinoma) [5, 6]. Hepatocellular cancer can also arise in the absence of cirrhosis.

NAFLD is considered a multisystem disease and patients are at high risk of developing extra-hepatic complications, including diabetes, cardiovascular disease and some type of cancers [7]. Conversely, in those with type 2 diabetes or cardiovascular disease, the incidence of NAFLD is increased, while the number and severity of metabolic risk factors increases the likelihood of inflammatory steatohepatitis.

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Cardiovascular disease is the number one cause of death in patients with NAFLD [5]. The gamut of hepatic and extra-hepatic diseases associations of NAFLD and NASH therefore poses a significant health, economic, and quality of life burden to both patients and their families, as well as on the health care systems [8]. Despite being a very common disorder, NAFLD is commonly under-diagnosed and more frequently diagnosed at the time of cirrhosis [9].

Though the pathogenesis of NAFLD is multifactorial, it is best conceived as a complex trait, shaped by the interaction between exogenous environmental factors (e.g. dietary intake and physical activity), the holobiome, individual genetic predisposition and epigenetic modulators, combining in unique ways in any individual to precipitate the development and progression of disease [10]. The reasons for inter-individual variability in disease expression it follows, is the integration of inputs from all these signals, one aspect of which is the genetic and epigenetic background [10, 11]. Identifying the underlying mechanisms and genetic risk associations of NAFLD is important for understanding disease pathogenesis, for developing new therapeutic targets and for diagnosis and risk stratification. In this review, we summarize data on the current state of knowledge with regard to the genetic and epigenetic mechanisms that govern or at least influence, the development of NAFLD.

NAFLD Is a Heritable Disease

Several lines of evidence estimated from twin-studies, familial-aggregation studies and epidemiological data suggest that NAFLD has a strong heritable component. Twin studies led to the estimation that more than half of the variability of liver fat and fibrosis can be accounted for by heritable factors [12] and the heritability of both conditions is mostly shared (~75.6%) [13]. Furthermore, familial aggregation studies found up to a 12.5-fold higher risk of NAFLD and advanced fibrosis in first-degree relatives of patients with cirrhosis related to NAFLD, compared to that of the general population, independent of multiple confounders [14]. Further, substantial inter-ethnic variability in NAFLD susceptibility is described, with highest prevalence in Hispanics, followed by Europeans and lowest in African-Americans [2, 15, 16]. As, discussed below variation in the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) gene has helped to explain some of this ethnic variability [15–17]. Notably, the estimated heritability level varies according to the ethnic population studied and study design [10].

Genetic Contributions to NAFLD

Our understanding of the genetic architecture and mechanistic underpinnings for NAFLD has exponentially broadened, primarily due to successful human genome-wide association studies (GWAS) [10]. Since the human genome was first sequenced

in 2003, followed by a GWAS on risk for age-related macular degeneration [18], there have been almost 3700 GWAS reported. These have agnostically identified thousands of genetic risk variants for various complex disorders and traits. Unlike Mendelian disorders caused by a single gene defect, most complex traits such as NAFLD are polygenic and rely on the additive and/or interactive effects of multiple genetic variants and environmental factors. The rapid pace of GWAS-based acquisition of knowledge has greatly facilitated the construction of a NAFLD genetic landscape. To date, these have revealed at least five novel variants in unique genes associated with NAFLD, namely *PNPLA3*, Transmembrane 6 superfamily member 2 (*TM6SF2*), glucokinase regulator (*GCKR*), Membrane bound O-acyltransferase domain-containing 7 (*MBOAT7*) and hydroxysteroid 17-beta dehydrogenase (*HSD17B13*) [10].

In 2008, the first GWAS for NAFLD used a custom chip of >9000 nonsynonymous variants in a cohort of more than 2000 ethnically diverse patients of European, Hispanic, and African American ancestries. Liver fat was quantified with high precision using proton magnetic resonance spectroscopy (MRS) [17]. The study identified a common nonsynonymous variant (rs738409 C > G p.I148M) in the phospholipase domain-containing protein 3 (*PNPLA3*) gene as robustly associated with increased liver fat across the various ethnicities [17]. This finding has since been replicated across diverse geographic regions and ethnicities and has extended to other phenotypes across the entire histological spectrum of NAFLD including NASH, fibrosis, HCC and NAFLD-related complications, as well as determining the chances of response to the beneficial effects of lifestyle intervention [19]. Significantly, the associations have extended to other liver diseases including alcoholic cirrhosis and viral hepatitis B and C [20–23]. A gene-environment interaction was observed for this variant, with obesity and measures of insulin resistance amplifying the genetic effect [24].

In humans, PNPLA3 is located on the long arm of chromosome 22 and encodes a 481-amino-acid protein and is widely expressed in multiple tissues, with highest levels in retina and liver. The protein is located on lipid droplets and the endoplasmic reticulum [25, 26]. *PNPLA3* is a multifunctional enzyme that regulates different aspects of lipid metabolism—encodes a triacylglycerol lipase, also called adiponutrin, which is involved in the hydrolysis of triacylglycerol in adipocytes and it also has acylglycerol O-acyltransferase activity [27, 28]. The specific SNP rs738409 C > G is peculiar in that it encodes an amino acid substitution from isoleucine to methionine at position 148 (I148M) near the catalytic domain and thus apparently abolishes or attenuates the hydrolase activity by ~0%, based on in-vitro studies [29]. As a loss of function variant, it thereby promotes triglyceride accumulation in hepatocytes [30, 31]. However, after more than a decade since discovery, the precise mechanisms by which PNPLA3 (148 M) promotes fatty liver remains ill defined. In murine models, mice with a knock-in introducing a methionine codon at position 148 of the Pnpla3 gene have normal levels of liver fat when fed normal chow. However, they have a two to threefold increase in hepatocyte fat compared to controls, when fed a sucrose-rich diet [32]. While, neither inactivation [33] nor overexpression [34] of PNPLA3 wild type in the livers of mice cause steatosis,

a recent study postulated a new mechanism and provided evidence that PNPLA3 interferes with adipose triglyceride lipase (ATGL) activity, the major lipase in the liver via interacting with comparative gene identification-58 (CGI-58) and thus promoting steatosis [35]. A retinyl ester activity for PNPLA3 has also been described in hepatic stellate cells (HSCs), with direct pro-inflammatory and fibrogenic roles for 148M in these cells [28, 36].

The second most studied variant is the nonsynonymous rs58542926 variant in the *TM6SF2* gene also identified from the Dallas Heart Study using the Illumina Human Exome chip for association with hepatic triglyceride content (HTGC), as measured by MRS. This variant explains the association signal with NAFLD observed at this multi-gene locus named *NCAN/TM6SF2/CILP2/PBX4*. The variant encodes for a Glu (E) to Lys (K) substitution at position 167 resulting in loss-of-function and reduced hepatic TM6SF2 expression [37–41]. In subsequent studies, the rs58542926 variant has been associated with fibrosis and HCC in NAFLD and with steatosis and liver injury from other diseases, including alcoholic cirrhosis and viral hepatitis B and C [37, 42].

In humans, TM6SF2 is located on chromosome 19 and encodes a protein of 351 amino acids. TM6SF2 is principally expressed in liver and small intestine and is located in the ER and the ER-Golgi complex [39, 43]. Current experimental evidence suggests that TM6SF2 regulates cholesterol synthesis and the secretion of lipoproteins [43, 44] and is involved in the enrichment of triglycerides to apolipoprotein B100 in the pathway of very low-density lipoprotein secretion from hepatocytes [44]. Interestingly, *TM6SF2*-rs58542926 is associated with a clinical paradox, wherein the T allele (Lys167) is associated with increased NAFLD and NASH but is protective for CVD. This association can be explained by the lowering effect of the risk allele on blood lipid levels.

Variation in the glucokinase regulator (GCKR) gene locus has been uncovered by a NAFLD-GWAS. GCKR regulates *de novo* lipogenesis by regulating the influx of glucose in hepatocytes [45–47]. The loss-of-function GCKR mutation (rs1260326) encoding the P446L protein variant is associated with a reduced ability to negatively control glucokinase in response to fructose-6-phosphate. This results in enhancement of lipogenic pathways and increased hepatic lipid accumulation [48, 49].

A fourth NAFLD risk variant in rs641738 C > T is located in the transmembrane channel-like 4 gene (TMC4)/MBOAT7 gene and is linked to the 3′ untranslated region (UTR) of *MBOAT7*. The variant was first identified by GWAS for alcoholic cirrhosis [42] and in subsequent reports has been shown to be associated with the entire histological spectrum of NAFLD [50]. This includes NAFLD-related HCC, particularly in patients without cirrhosis [51] and liver injury in viral hepatitis (both hepatitis B and C) [52, 53]. The variant correlates with downregulation of hepatic MBOAT7 expression and activity [50, 54].

Finally, a protein-truncating variant in the *HSD17B13* gene (rs72613567:TA) has been uncovered by an exome-wide association study to be associated with decreased serum liver enzyme levels (alanine transaminase (ALT) and aspartate aminotransferase (AST)) and NASH [55] The variant has recently been shown to be protective of HCC development in patients with ALD [56]. Subsequently, a couple

of other variants in *HSD17B13* were described, namely rs62305723 (encoding a P260S mutation) [57] and rs143404524 that can lead to premature truncation of the protein via introducing a frameshift at codon at position 192 [58].

The HSD17B13 gene is highly expressed in liver and is a lipid droplet protein with retinol dehydrogenase activity [57]. However, the exact mechanism whereby HSD17B13 protects against progressive liver damage in NAFLD has not been elucidated; it appears to be independent of liver fat accumulation [55]. Data from Hsd17b13 KO mice suggests opposite results to humans, as HSD17B13 disruption triggers hepatic steatosis and inflammation.

Other Genetic Loci Associated with NAFLD Development and Progression

Several other genetic variants implicated in the regulation of insulin signalling, lipid metabolism, oxidative stress, adipokines and myokines, immune responses, inflammation and fibrosis have been reported to be associated with NAFLD development and progression [10, 11, 59]. Overview on these variants is provided in next section.

Insulin Resistance

Consistent with a pivotal role for insulin resistance in the pathogenesis and progression of NAFLD, polymorphisms of genes related to insulin signalling have been associated with fibrosis in NAFLD. A gain-of-function polymorphism (rs1044498) in the ectonucleotide pyrophosphatase/phosphodiesterase (*ENPP1*) is associated with the severity of fibrosis in NAFLD via facilitating the ENPP1 glycoprotein interaction with the insulin receptor. Similarly, the loss-of-function insulin receptor substrate 1 (IRS1) G972R variant (rs1801278) was demonstrated to be associated with attenuation of hepatic insulin signaling and an increase in the severity of fibrosis [60]. Likewise, the rs2954021 variant in Tribble-1 (TRIB1), another regulator of insulin signalling has been reported to be associated with the development and severity of NAFLD, via its altered expression [61, 62]. TRIB1 has been implicated in the regulation of microsomal triglyceride transfer protein expression [63] and de novo lipogenesis [64].

Adipokines and myokines are involved in NAFLD pathogenesis. The rs3480 variant in the irisin fibronectin type III domain-containing protein 5 (*FNDC5*) locus has been linked with steatosis severity in NAFLD, via a miR-135a-5P-mediated mechanism controlling FNDC5 mRNA stability [65]. Of relevance, miR-135a maps to an overrepresentation of insulin signaling and type 2 diabetes pathways and inhibits insulin signaling by targeting the insulin receptor substrate 2 (IRS2) [66]. Multiple lines of evidence in humans, in-vitro and in vivo mouse models suggests that irisin has anti-steatotic effects and a favourable metabolic

impact on NAFLD [65, 67, 68]. Multiple variants (at position −11377C/G and +45T/G) in the adiponectin gene have also been associated with the severity of inflammation and reduced adiponectin levels in patients with NAFLD [69].

Nuclear Receptors

Nuclear receptors are pivotal in the regulation of hepatic lipid metabolism, and thus, are obvious therapeutic targets for NAFLD. However, conflicting results have been reported regarding the association of peroxisome proliferator-activated nuclear receptors to the severity of hepatic steatosis and liver damage [70, 71]. Other candidate genes and variants include the rs13412852 polymorphism in the *LIPIN1* (a phosphatidate phosphatase gene) gene that is associated with protection from NAFLD and metabolic syndrome via increasing LIPIN1 expression [72], and rs56225452 in the promoter of fatty acid transport protein 5 (*FATP5*) gene. The latter has been associated with the severity of steatosis in NAFLD [73].

The Mitochondria and Oxidative Stress

A rs695366 variant in the promoter region of the Uncoupling Protein 2 (*UCP2*) gene has been associated with reduced susceptibility to NASH through increased UCP2 expression [74]. Uncoupling proteins control mitochondrial redox generation and mitochondrial energy dissipation but is also implicated in fatty acid export from the mitochondria [75]. Likewise, the manganese superoxide dismutase (SOD2) rs4880 SNP is associated with reduced fibrosis in patients with NAFLD perhaps by regulating SOD2 mitochondrial import and antioxidant activity [76, 77]. These findings are in line with a pivotal role for mitochondria-derived oxidative stress in the progression of liver injury.

Innate Immunity, Inflammation and Fibrosis

Genetic variation in the interferon (IFN)-λ3/IFN-λ4 region regulates innate immunity [78, 79] and has been shown to be associated with hepatic inflammation and fibrosis in patients with viral hepatitis and NAFLD [80–83]. Similarly, the IVS1-27G > A (rs3750861) variant in Kruppel-like factor 6 (KLF6) attenuates activation of hepatic stellate cells and thereby reduces fibrosis progression in NAFLD via modulating alternative splicing [84]. Notably, the KLF6 variant also influences fasting blood glucose, the expression of glucokinase, and lipogenesis. Finally, a variant rs4374383 G > A in the MER proto-oncogene, tyrosine kinase (*MERTK*) gene has been implicated in the regulation of efferocytosis (which describes the

process of removal of apoptotic or necrotic cells by the phagocytic cells) in macrophages and is potentially important for hepatic stellate cell activation. This variant demonstrates a protective association and reduces fibrosis in patients with chronic HCV infection [85] and NAFLD. The variant is also associated with reduced 9-year incident NAFLD and T2DM [86, 87].

Pleiotropy

As NAFLD is a multi-system disease, an interesting angle is to explore its shared genetics with other related metabolic disorders to identify potential pleiotropic effects of NAFLD-related variants. The term 'Pleiotropy' refers to the phenomenon whereby a gene or genetic variant affecting several traits [88]. Pleiotropy is common in the human genome [89, 90], with a recent estimation that nearly half the genes identified in the GWAS catalogue are pleiotropic [91], and tends to be more prevalent for variants associated with diseases in the same broad category [92, 93]. Recently, phenome wide association studies (PheWASs) have become a complimentary tool to GWAS, to investigate pleiotropy [94]. Of relevance, a recent twin study suggested substantial shared gene effects between hepatic steatosis and related metabolic traits including lipid and glycaemic profiles and blood pressure [13].

Along this vein, recent exome-wide association studies have demonstrated that variants in *TM6SF2* and *PNPLA3* are associated with reduced lipid levels and coronary artery disease risk, but with enhanced risk of fatty liver and type 2 diabetes [95]. Likewise, a recent PheWAS demonstrated that *HSD17B13* rs72613567 is associated with higher platelet counts, likely a reflection of its association with chronic liver disease [55]. Another PheWAS identified pleiotropic effects for *PNPLA3* rs738409, including with elevated liver enzymes, an increased risk of type 2 diabetes mellitus and nonsteroidal anti-inflammatory drug (NSAIDs) induced liver injury. This same variant has been associated with a reduced risk for acne, gout and gallstones, high cholesterol and intake of cholesterol-lowering medications [96].

Epigenetic Factors and NAFLD

Epigenetic changes refer to heritable phenotypic variation and changes in gene expression that operate outside of changes in the DNA sequence. Epigenetics represents a plausible link between genes and environmental factors, since they are modulated by environmental stimuli and can explain part of the missing heritability of complex traits such as NAFLD. Epigenetics determines how genes are expressed and their dysregulation is a hallmark of many diseases, including NAFLD [10]. The most described epigenetic modifications include: (1) histone modifications; (2) DNA methylation; (3) microRNAs; and (4) chromatin remodelling, and they will be discussed in the subsequent sections.

DNA Methylation and Chromatin Remodelling

DNA methylation is a critical form of epigenetic regulation that refers to the methylation of cytosine bases clustered in so-called CpG-islands that orchestrate gene expression networks for many biological processes. Aberrant DNA methylation of gene promoters (hypermethylation) and subsequent transcriptional silencing is a potent contributor to diseases, including NAFLD and fibrosis [10, 97]. DNA methylation is often altered in NAFLD and genome-wide screening studies of liver biopsies from patients has demonstrated a widely altered methylation signature of hepatic DNA. Loci include those involved in the methylation process, certain metabolic pathways, inflammation, and fibrosis, suggesting that the epigenetic changes are important for the pathogenesis of NAFLD and the progression to fibrosis [98, 99]. Similarly, peripheral blood-derived DNA methylation signatures have been associated with hepatic fat [100]. Consistently, mice lacking the master epigenetic regulator methyl CpG binding protein 2 (MeCP2), demonstrate attenuated fibrosis in multiple tissues, including liver, lung and heart [101, 102]. There is evidence also that gene expression of PNPLA3 might be regulated by DNA methylation [103].

Additional evidence for a crucial role for epigenetic mechanisms in modulating the susceptibility to NAFLD can be inferred from the role of foetal metabolic programming of liver fat [104, 105]. An adverse intrauterine environment including a high fat diet in experimental animal models was sufficient to trigger metabolic maladaptation that is accompanied by functional alterations of foetal hepatic DNA methylation and histone modifications. In turn, these changes favoured the development of NAFLD in the offspring. Notably, some of the changes persist up to 5 weeks of age [106, 107]. Another study also suggested a potential intergenerational adaptation in the hepatic wound-healing, in which ancestral history of liver fibrosis seems to attenuate fibrogenesis in next generations [108].

Such data clearly indicates that the earliest origins of NAFLD and other metabolic diseases may reside in *in utero* experiences. Thus, perhaps the critical time for reestablishment of genome-wide epigenetic profiles might occurs in early embryogenesis. Though data in humans are limited, indirect evidence supports this notion. In one study, increased methylation of peroxisome proliferator-activated receptor γ coactivator 1 (PGC1), a central regulator of multiple aspects of energy metabolism, including mitochondrial biogenesis, oxidative phosphorylation and insulin sensitivity in liver from NAFLD patients, correlated with insulin resistance phenotype and mitochondrial biogenesis [109]. In another report, maternal pre-gestational BMI was associated with methylation of the PGC1 promoter in newborns [110]. Added evidence comes from animal models demonstrating that maternal high fat feeding leads to a decrease in offspring PGC1α mRNA expression and hepatic mitochondrial content, as seen in adult humans with NAFLD [111].

Notably, as established epigenetic landmarks are relatively stable and heritable through mitosis, a woman who has modifiable risk factor induced epigenetic changes can alter outcomes for three generations: herself, her unborn daughter and her daughter's reproductive cells. Some animal studies have suggested that a sub-

optimal grand-maternal diet increased intra-abdominal fat mass in granddaughters [112]. Interestingly even paternal diet and prediabetes increases the risk of diabetes in the off spring [113].

Based on the above data, a beneficial rewriting of the early-life epigenome may lead to advantageous outcomes such as disease prevention. Since epigenetic mechanisms are frequently regulated by environmental factors, maternal diets and early, life style interventions and exercise may play an important role in influencing early-life epigenetic reprogramming processes leading to phenotypic changes and altered risk in the offspring [114]. In this regard, the pattern of infant nutrition and maternal obesity have been shown to influence the risk of NAFLD in adolescents. For example, breast-feeding for at least 6 months and normal pre-pregnancy BMI can reduce the risk of NAFLD development during adolescence in offspring [115]. Similarly, another study reported that lactation duration of >6 months is associated with lower risk of NAFLD in mid-life [116]. In animal models, exercise has reversed maternal high-fat, diet-induced metabolic dysfunction and hypermethylation of the Pgc-1α gene in the offspring [117].

Epigenetics findings can have important translational implications. A previous study suggested that plasma DNA methylation of PPARγ has diagnostic utility, with an AUROC of 0.91 to non-invasively stratify severe liver fibrosis (F3-F4) in patients with NAFLD [97]. On the other hand, methylation changes in NAFLD have also been demonstrated to be partially reversible following bariatric surgery [98]. This provides a strong rationale for epigenetic modulation to treat NAFLD in order to restore the normal (healthy) epigenetic landscape. This outcome is supported by studies demonstrating the ability of pharmacological DNA methylation inhibitors to suppress fibrosis progression [118]. Current interest in epigenetics as targets for therapy is evident from the exponential growth in the epigenetic drugs market, with it being valued at US$4.63 billion in 2017, and predicted to reach $16.5 billion by 2026 [119]. While nucleotide analogues are used as demethylating therapies for diseases such as myelodysplastic syndrome, they are nonspecific and their utility is limited to second line, due to side effects [120]. Very recently, repurposing CRISPR technology for methylation editing has provided hope for targeted methylation editing with limiting off-targets effects [121–123]. This approach is yet to be explored in NAFLD.

Non-coding RNAs

Another level of epigenetic regulation is based on non-coding RNAs that include MicroRNAs (miRNAs) and Long non-coding RNA (lncRNA) that regulate gene expression at both the transcriptional and post-transcriptional level [10]. miRNAs are small non-coding RNA transcripts of ~22 nucleotides and the human genome contains over 2000 miRNAs that regulate gene expression (mainly by inhibition) at the post-transcriptional level [124]. miRNAs are frequently studied in NAFLD and a recent meta-analysis demonstrates that some miRNAs, namely miRNA-122,

miRNA-34a and miRNA-192 can serve as biomarkers to distinguish NAFLD and NASH severity. miRNA-122 for example could delineate NAFLD from healthy controls, however the diagnostic accuracy appears modest, and the results appear to differ between studies and between circulating and hepatic miRNA expression patterns [125]. More standardisation in the used techniques to measure miRNA and the use of sensitive tools such as droplet digital PCR could help to improve the detection of miRNAs and verify their clinical utility, as recently suggested [126].

miRNAs regulate multiple biological pathways involved in the pathogenesis of NAFLD [125]. miR-122 is the most studied and abundantly expressed hepatic miRNA (70% of total abundance) and is implicated in multiple pathways in the progression of NASH. Downregulation of miR-122 enhances lipogenesis in *in-vitro* models [127]. Consistently, hepatic deletion of miR-122 is associated with spontaneous development of NASH and subsequent progression to HCC via increased lipogenesis and impaired lipid secretion in mouse models [128]. Reduction of miR-122 is also implicated in fibrosis by inducing multiple fibrotic pathways, such as mitogen-activated protein kinase 1 (MAPK1) and hypoxia-inducible factor 1-α (HIF1α) [129].

Data on the role of lncRNAs in NAFLD is limited. A recent study conducted lncRNA gene expression profiling in 82 liver samples from individuals with NASH, simple steatosis, and healthy controls, followed by replication in a cohort of 44 liver biopsies. That study identified a liver-specific lnc18q22.2 to be significantly elevated in livers from patients with NASH [130]. Another recent study demonstrated that a brown fat-enriched lncRNA 1 (Blnc1) is robustly elevated in NAFLD in mice, while liver-specific inactivation of Blnc1 abrogates high-fat diet-induced hepatic steatosis and NASH [131]. The role of lncRNAs in NAFLD remains to be characterised in large cohorts.

Clinical and Translational Implications

Over the last decade, our understanding of the genetic and epigenetic basis for NAFLD and NASH has evolved, fuelled by GWAS and exome wide association studies. However, the number of discovered variants remains a handful, compared to that discovered for other related conditions such as diabetes, the lipid profile and CVD. Further, known NAFLD variants explain only a fraction of its heritability. A large block or missing heritability exists and global collaborative efforts have now begun to consider other types of genetic variation including copy-number variants (CNVs). There are known mechanisms by which CNVs can be associated with disease, and elucidation of interaction effects, whether between genes or with the environment can help fill the missing heritability. However, it has to be recognized that considerably less genetic variation has been discovered for NAFLD than is expected, given the well-validated heritability estimates, or for that matter, in comparison with other related disorders such as diabetes, the lipid profile and CVD.

The term 'polygenic' refers to the genetic architecture underpinning variations in a disease between different individuals in a population. However, from a clinical perspective, what does the risk mean for an individual and what will be the translational implications of genetic findings for assisting in risk prediction and enabling personalized and preventative medicine? Clearly, each individual will carry a unique combination of sets of a number of alleles that increase and others that decrease disease risk. Therefore, the large odds ratios and statistical significance of the identified NAFLD variants do not necessarily imply clinical relevance and the effect of any genetic risk variants might be of small effect size and unlikely to be clinically meaningful. Thus, developing polygenic scores and algorithms for the cumulative effects of multiple loci and perhaps the incorporation of clinical variables, provides more robust hope for the development of accurate algorithms with higher discriminatory performance. For example, polymorphisms in the *IFNL3* gene together with clinical variables can be incorporated into a predictive score for multiple liver diseases [81]. Similarly, a combination of genetic risk variants (i.e., in *PNPLA3*, *TM6SF2*, and *MBOAT7*) and clinical variables has been used for the prediction of HCC in NAFLD. Notably, considering not only the top GWAS hits, but also other biologically plausible variants can aid in improving clinical predictive models. As an example, a variant in the *FNDC5* gene has been found to have an independent but additive effect to *PNPLA3* and *TM6SF2*, with higher association for hepatic steatosis [65].

As a field, we are gaining momentum moving from studies of association to understanding biological function and thereby closing the gap to translation and new therapeutics [78]. The path from GWAS to the underlying biology however may not be straightforward, as any association between a risk variant at a genomic locus and a trait is not directly informative with respect to the causal variant. However, there is increasing hope that accumulating genetic discoveries including for NAFLD can help to modernise the drug development process and aid in the elucidation of more efficient therapeutic targets [132]. Some of the successful targets for drugs currently in use have been retrospectively substantiated by GWAS, such as the lipid-lowering proprotein convertase subtilisin/kexin type 9 (PCSK9)-inhibitors, among the top identified risk variants for blood lipids. Even though we are at an early phase, applying the knowledge from phenome-wide association studies, will likewise allow us to investigate for associations between a specific genetic variant/s, and a wide range of phenotypes. In turn, this will represent an enormously attractive approach to aid and accelerate drug development, and drug repurposing [132].

In conclusion, given the global burden of NAFLD and NASH, efforts will continue in earnest to develop accurate non-invasive diagnostic and prognostic biomarkers and novel therapeutic targets. Genetic discoveries that have been made, and future discoveries, will accelerate the path to this future goal.

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Chapter 4
Geometry of Nutrition: Nutrients and NAFLD Progression

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Introduction

Several questions remain regarding NAFLD management. However, it is certain that we should control the epidemic obesity. It is important to work on lifestyle interventions, specifically on diets. In fact, Younossi [1] encourages following a diet lower in both fat and sugars. Studies have shown that a healthy diet and weight loss (at least 7%) in the early stages of NAFLD [2] might prove sufficient to control disease progression. Moreover, a recent meta-analysis corroborates that exercise coupled with dietary intervention is the most effective treatment [3]. Thus, it seems the primary role of dietary intervention is improving aminotransferases [4]. However, despite consolidate evidence of a diet intervention being effective mitigation, the importance of the extent and the composition of the diet is less known. Additionally, in many occasions, patients fail to adhere to the diet intervention. Thus, it is necessary to establish simple nutritional guidelines targeting the disease mechanisms and slowing down disease evolution.

Role of Macronutrients on NAFLD

Several studies have shown the role of specific macronutrients in the onset and progression of NAFLD. Macronutrients such as saturated fatty acids, trans-fats, simple sugars (sucrose and fructose) and animal proteins harm the liver. As suggested by

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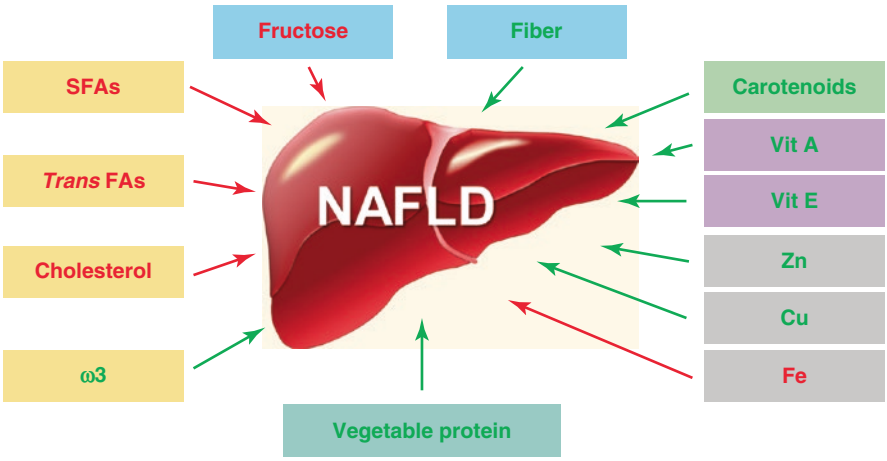

Fig. 4.1 Macro and micronutrients affect in different ways NAFLD development. Nutrients are important contributor factors affecting NAFLD pathogenesis. Green arrows represent nutrients that prevent NAFLD. Red arrows represent nutrients that promote NAFLD. *SFAs* saturated fatty acids, *Trans FAs* trans fatty acids, *ω3* omega-3 fatty acids, *Zn* zinc, *Cu* copper, *Fe* iron

Musso et al. [5], these modulate the liver's triglyceride accumulation, which affects insulin sensitivity and postprandrial triglyceride metabolism. Moreover, monounsaturated fats (MUFAs), polyunsaturated (PUFAs) omega-3-fats, plant-based proteins and dietary fibres seems to be beneficial for liver health [4] (Fig. 4.1).

Role of Fats

Despite the general consensus that saturated fats intake should be reduced, the issue of the fatty acid compositions of diets remain controversial. Saturated fat intake is related with an impaired glutathione metabolism and increased oxidative stress, leading to NAFLD progression. However, the effects of saturated fats seem to depend on the patients' genetic background [6]. The specific effects of trans-fats on the human liver has not been properly evaluated. In mice models, chronic (over 4 months) trans-fat-high diets induce non-alcoholic steatohepatitis (NASH) [7] and hepatocellular carcinoma [8]. Alferink et al. [9] showed how trans-fat intakes were related with the increased probability of NAFLD development in humans. In the case of MUFAs, studies show different, sometimes opposite, conclusions. Cortez-Pinto et al. [10] found a higher MUFAs consumption in NAFLD patients. Moreover, Alferink et al. [9] did not find any beneficial effect of MUFA intake on NAFLD. Finally, Rietman et al. [11] found that MUFA consumption was positively associated with a higher fatty liver index. However, Zelber-Sagi et al. [12] in the context of a cross-sectional sub-sample of the Israeli National

Health and Nutrition Survey, were unable to find an association between MUFAs' intake and NAFLD. On the contrary, some studies suggest that such intake could improve fatty liver damage. Furthermore, an isocaloric diet enriched in MUFAs, as compared to a diet higher in carbohydrates and fiber, could induce a significant reduction of liver fat, as found in a controlled randomised study in type 2 diabetic patients [13]. Moreover, the consumption of 20 g/day for 12 weeks, in hypocaloric diets attenuated a fatty liver grade in patients with NAFLD. However, the effects of polyphenols present in olive oil and the importance of the hypocaloric diet should be considered [14]. Additionally, a 6-month intervention study, with oils rich in MUFAs, in NAFLD patients significantly reduced fatty livers [15]. PUFAs primarily include omega-3 and omega-6 fats. Nowadays, it is well known than besides the total PUFA intake, the ratio of omega-6 to omega-3 fats plays an important role in increasing the prevalence of chronic metabolic diseases. Traditionally, this ratio has been 1:1 for humans. Moreover, it has actually been modified in favour of omega-6 fats [16]. Omega-6 fatty acids are represented by linoleic acid (LA) and omega-3 fatty acids by alpha-linolenic acid (ALA). LA is metabolised to arachidonic acid (AA), while ALA is metabolised to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The eicosanoid metabolic products from AA (prostaglandins, thromboxanes and leukotrienes) are proinflammatory, prothrombotic and proaggregatory. On the other hand, EPA and DHA modulate the liver's lipid composition, increasing anti-inflammatory mediators and decreasing insulin resistance [17]. In fact, low EPA and DHA liver values could tilt the balance towards liver fatty acid lipogenesis, instead of fatty acid beta-oxidation. Thus, an unbalanced omega-6 to omega-3 ratio in favour of omega-6 PUFAs contributes to the prevalence of chronic metabolic diseases. A systematic review and meta-analysis of controlled intervention studies on the effects of omega-3 PUFAs in NAFLD patients [18] indicate that supplementation with omega-3 decreases the liver's fat content and steatosis score, being well tolerated. However, the effects of supplementation on improving severe liver injury markers, such as inflammation and fibrosis is not well established [19]. Regarding the significance of cholesterol in NAFLD development, most studies have focused on fatty acids and triglycerides; thus, the contribution of dietetic cholesterol is not clear enough. Cholesterol is absorbed from the diet in the jejunum; additionally, it is synthesized in various tissues (for example, hepatocytes). Once absorbed, cholesterol is transported to the liver as chylomicrons and their remnants. Some nutritional studies suggest that high-cholesterol diets are involved in NAFLD development [5]. However, the same studies show that patients had high fat intakes. An interesting finding is that cholesterol intake was significantly higher in non-obese NAFLD patients than in obese ones. The proposed mechanisms is that excess cholesterol intake can upregulate LXRα expression, which in turn activates fatty acid synthesis. Thus, cholesterol overload itself can stimulate the NAFLD development, despite the total calorie intake being within the normal range [20]. Additionally, non-obese mice were fed a high cholesterol diet, but the ones with a normocaloric diet developed NAFLD [21].

Role of Carbohydrates

During the last two decades, much evidence has been accumulated showing the adverse metabolic effects owed to the high consumption of simple carbohydrates (CHO). Studies cast doubts upon the real role of monosaccharides and disaccharides in NAFLD. It has been observed that NAFLD patients had a significantly higher glucose intake per 1000 kcal [22]. Moreover, high carbohydrate diets (>1000 kcal) during 3 weeks in obese patients induced a tenfold greater relative change in liver fats. Thereafter, the subsequent change to hypocaloric diets for 6 months normalised liver fat values [23]. Contrarily, Rietman et al. [11] found an inverse correlation between monosaccharides, disaccharides and NAFLD. Soft drink consumption was significantly associated with NAFLD. These are a blend composed of 55% fructose. Additionally, fructose has increasingly been used as a sweetener since the 1960s. The liver is the primary site of fructose metabolism, with an oxidation of almost 60% fructose ingestion. Furthermore, fructose metabolization by the liver is much higher than that of glucose. The hepatic metabolism of fructose stimulates liver de novo lipogenesis (DNL), increasing liver fat [24]. This induced DNL is mediated by the activation of the carbohydrate responsive transcription factor carbohydrate response element binding protein (ChREBP) [25]. In patients with NAFLD, 26% of liver fat comes from DNL and 15% from the diet [26]. These are independent of insulin. Moreover, the fructose phosphorylation in the liver consumes ATP, which induces ADP accumulation. This ADP acts as the substrate for uric acid formation. These factors promote hepatic oxidative damage and lipid peroxidation [26]. Human studies (cross-sectional and retrospective case-control) have demonstrated an association between fructose intake and NAFLD [27–29]. There is a systematic review and meta-analysis of controlled feeding trials that conclude the isocaloric exchange of carbohydrates for glucose does not induce NAFLD. However, when fructose is the source of a hypercaloric diet, NAFLD patients have increased liver fats and plasma alanine aminotransferases [30]. Furthermore, Abdelmalek et al. [31] showed that in adult patients with NAFLD, an increase in fructose consumption was associated with reduced liver steatosis, but increased fibrosis and ballooning. This was achieved after controlling for age, gender, body mass index (BMI) and total calorie intake. The role of non-digestible carbohydrates (fibre) in NAFLD has also been explored, although it is not studied as in depth. A decrease in fiber consumption is related with NAFLD [10, 22, 32]. However, the mechanisms responsible for this association are poorly understood. The proposed rationale is that low fibre intake, along with other dietary patterns, induce dysbiosis. The alteration in microbiota causes endotoxemia and systemic inflammation, enhancing insulin resistance, liver inflammation and damage. Prebiotic fibres could improve such microbiota composition. An alteration of gut microbiota has been observed in NAFLD patients. Additionally, prebiotics' intake is shown to improve the liver phenotype in NAFLD patients [33].

Role of Proteins

The role of protein intake on NAFLD development is difficult to establish. Studies provide no evidence for or against. Few studies found a significantly higher protein intake in NAFLD patients [10, 22], while others found no changes in protein intake in NAFLD patients as compared to healthy people [5, 32]. Lastly, few studies show that a high protein intake improves NAFLD [34]. It has been suggested that the difference in obtained results is due to the origin of the protein source; however, this remains unclear. For example, Rietman et al. [11] found an inverse relationship between vegetable protein intake and NAFLD. Additionally, this study observed that animal protein intake was positively associated to NAFLD. The rationale proposed for protein intake and NAFLD is that a high protein intake activates the mammalian target of rapamycin complex 1 [35], which in turns deteriorates the metabolic control and increases liver triglycerides [36]. On the other hand, Markova et al. [34], in a prospective study of patients with type 2 diabetes and NAFLD, employing 2 isocaloric high-protein diets noted that high protein diets (either animal or plant) significantly reduced insulin resistance, liver fats and necroinflammation. Since the cohort used was over 60 years, the authors suggest that the effects are age related. The mechanism proposed was modifications in the lipolytic and lipogenic pathways in adipose tissues.

Role of Micronutrients on NAFLD

Micronutrients are important for NAFLD development. Various methods allow to achieve this statement. First, decreased serum levels of vitamins are frequently present in patients with NASH [37]. Second, there are studies that boost the consumption of micronutrients to prevent and treat NAFLD [38]. Third, some authors consider micronutrients to contribute to NAFLD physiopathology [39]. The question is thus where micronutrients can be involved in NAFLD pathogenesis. It is well known that lipid accumulation occurs in NAFLD. These bioactive lipids can induce lipotoxicity and oxidative stress. The consequent response of liver nonparenchymal cells induce inflammation and fibrosis. Finally, micronutrients have antioxidant and anti-inflammatory properties.

Till date, the micronutrients involved in NAFLD are zinc, cooper, iron, vitamins A, C, D and E and carotenoids [39]. The proposed mechanisms of action are through their antioxidant, antifibrotic, immunomodulatory and lipoprotective effects (Fig. 4.1).

Minerals and NAFLD

NAFLD patients have been found deficient in zinc [40]. In rodent models, it has been proposed that zinc deficiency increases lipotoxicity-induced oxidative stress [41].

Serum and liver levels of copper are decreased in NAFLD animal models. Moreover, such low levels are associated with a worsening of the disease (hepatic steatosis and higher liver weight) [42].

An iron excess is involved in liver lipid peroxidation and NAFLD severity [43]. The signalling pathways affect hepcidin (an iron regulatory protein) and ferroportin (an enterocyte basal membrane iron transporter responsible of iron homeostasis) [44]. Excess liver iron blunt liver lipid homeostasis, increases inflammasome and inflammatory cytokines [45]. There exist data showing an association between liver iron levels and NAFLD [46]. Finally, Maliken et al. [45] have showed a direct relationship between an increase in the apoptosis in liver reticuloendothelial cells and their iron accumulation in NAFLD patients.

Vitamins and NAFLD

Lipid soluble vitamins have been linked to NAFLD. Liver is the primary storage site of vitamin A (mostly the quiescent hepatic stellate cells). In fact, when activated, these cells lose their vitamin A content [47]. It has been observed that NAFLD patients have decreased levels of serum retinoic acid; furthermore, their retinoic X receptor α expression is reduced. Moreover, patients have shown an increased liver metabolism and insulin resistance, associated with serum retinoic acid concentrations. Additionally, their NAFLD activity score was correlated with the serum retinoic acid levels [48]. Although the studies indicate the beneficial effects of vitamin A, some concerns remain considering supplementation, as vitamin A has many other effects. The role of vitamin C in NAFLD is controversial. In children, a weak association between low vitamin C levels and the disease is observed [49]. In an adult male population, there exists a significant positive association between low vitamin C intakes and NAFLD [50]. However, other studies show no association [51]. NAFLD patients showed lower serum levels of vitamin D [52]. Furthermore, people with higher plasma vitamin D levels had a lower risk of NAFLD [53]. Thus, vitamin D levels are inversely correlated with NAFLD, independent of other causes [54]. However, there exist a debate whether vitamin D deficiency is the precursor or a consequence of liver disease. For example, NAFLD patients orally supplemented with vitamin D_3 fortnightly for 4 months and did not show improvements in liver transaminase levels and liver inflammatory markers [55]. The effects of vitamin D could be owed to its function on immune modulation, cell differentiation, proliferation and inflammatory response. Serum concentrations of vitamin E (alpha-tocopherol) depend on the liver, which takes up the nutrient after the various forms are absorbed from the small intestine. Treatment with vitamin E is shown to decrease transaminase levels and liver lobular inflammation, improve liver histology and reduce steatosis [56]. In fact, vitamin E supplementation is a common practice in NAFLD patients [57]. Vitamin E has antioxidant effects and NAFLD patients present with increased oxidative stress; thus, higher vitamin E intake might counteract the lipid peroxidation. In children, decreased levels of vitamin E intake is related

to higher levels of hepatic steatosis [49]. However, vitamin E supplementation could have different side effects, such as an increase of the likelihood of certain types of cancer or the risk of hemorrhagic stroke. Several meta-analyses of randomised trials have showed a small but significant increase in all-cause mortality [58, 59]. This raises the question of the safety of large vitamin E doses.

Lastly, carotenoids plasma levels are low in NASH patients [60]. Moreover, there exists an inverse association between serum carotenoids levels and NAFLD prevalence [61]; these levels are associated with the improvement of the disease [62]. In dietetic NAFLD-induced rodent models, β-carotene supplementation reduces liver oxidative stress, steatosis and liver damage [63]. It is proposed that carotenoids accumulates in the liver inducing a down-regulation of lipogenic and fibrogenic genes, decreasing inflammation and enhancing lipolysis [64].

As a final remark on the role of vitamins, it is important to note that there can be interactions between different vitamins and between vitamins and macro/micronutrients. Some studies consider micronutrients mixes could be considered for NAFLD treatment [65]. Additionally, the dissection of specific micronutrient contribution is difficult, since human diets are complex, variable and sometimes do not replicate experimental dietary models. Thus, it is difficult to recommend diets with specific micronutrients composition.

Foods Groups, Dietary Patterns and NAFLD

Foods Groups and NAFLD

The National Institutes of Health defines nutrients as chemicals compounds present in food used by the body to function properly and maintain its health. People eat foods and the food forms a part of dietary patterns. In fact, the macro- and micronutrients that integrate the food interact among themselves in a highly complex manner to give rise to diet properties. Hence, the analysis of food groups' intake is important, reflecting a more physiological situation. Till now, we have focused on the effect of the different groups of nutrients in NAFLD, trying to understand their individual role in the disease's development. However, it is important to broaden the perspective and consider the role of what people eat in NAFLD pathophysiology.

Generally, there is a relationship between various food groups' intake, diets and NAFLD. However, it is difficult to establish a clear conclusion owed to some major limitations like a different study design, no control groups, a low number of patients, different types of patients included in the studies, no control of confounder factors, the diagnostic methods of NAFLD and other problems. Nevertheless, there is a general consensus that the intake of a variety of food is important to prevent NAFLD development [66].

Most guides to healthy eating establish five main food groups: (1) bread, cereals, rice, pasta, noodles and other grains; (2) vegetables and legumes; (3) fruits; (4)

milk, yoghurt, cheese and alternatives and (5) lean meat, fish, poultry, eggs and nuts. A balanced diet should include a variety of foods from each of these groups daily. A poor dietary composition is an important factor in NAFLD progression.

Studies portray that many NAFLD patients overconsume fructose, red meat, processed meat and saturated fats [12, 67], such as sodas, frozen junk foods, juices, whole fat dairy foods, fatty snack foods, take away foods, cakes and biscuits (Fig. 4.2). In this regard, several studies have showed that NAFLD patients have consumed high levels of protein sourced from meats, specifically red meats [12, 68]. Furthermore, the same relationship has been established with processed meats [69]. A recent study of Zelber-Sagi et al. [70] showed that patients with NAFLD had a higher intake of red and processed meats. They established an association between such meats (a weekly consumption of over 2 servings of 100 g), NAFLD and insulin resistance. The effect was independent of saturated fat and cholesterol intake. Additionally, cooking the meat at high temperatures for a long duration could be an important factor. In the case of proteins, the source of protein is important. For example, when protein intake is sourced from fish high in omega-3 (mackerel, salmon, tuna, haddock, trout, cod, herring, sardines and anchovies), this often tends to decrease NAFLD risk [68] (Fig. 4.2). Similarly, Allard et al. [71] observed that NASH patients consumed PUFAs below the recommended levels and had lower concentrations of liver omega-3s. A recent meta-analysis identified several relationships between food group intakes and NAFLD [72]. NAFLD patients consumed lesser cereals, grains, fruits and vegetables than healthy subjects (Fig. 4.2). Contrarily, NAFLD patients had a higher intake of cooking oils, candies, pastries, desserts, salty food, spicy food, sauce, dressings and soft drinks (Fig. 4.2). Another review study indicated that the consumption of soft drinks can increase the prevalence of NAFLD, independent of metabolic syndrome [73]. Furthermore, artificially

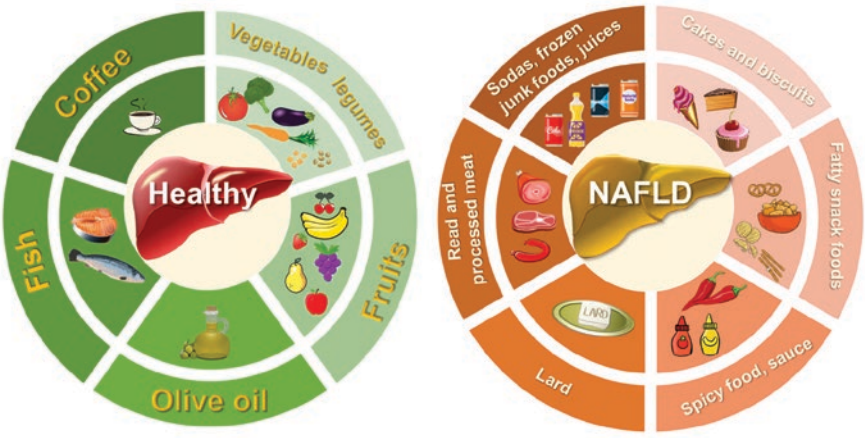

Fig. 4.2 NAFLD food chart. Left panel: NAFLD patients have a low intake of the foods represented. Right panel: NAFLD patients have a high intake of the foods represented. Foods are grouped in the most common food groups

sweetened diet soft drinks, although a healthier alternative to soft drinks, do not guarantee protection against NAFLD [74]. Finally, Mirmiran et al. [72] found that studies regarding the consumption of dairy products showed inconclusive results. As people consume different amounts of various food groups, in some cases, the relationships already mentioned are not so clear. Tajima et al. [75] found a positive association between rice consumption and NAFLD prevalence in women, but not in men. Coffee is one of the most commonly consumed hot beverage. Studies suggest that its regular consumption could a have a protective role against NAFLD and fibrosis [76] (Fig. 4.2). However, other studies show conflicting results regarding coffee's effect on steatosis [66]. Nevertheless, the compounds in coffee involved in its beneficial effects are presently unknown, although caffeine and its polyphenolic fraction may exert an important role. Additionally, extra virgin olive oil exerts its healthy effects through two principal components: MUFAs (particularly oleic acid) and phenolic compounds (Fig. 4.2). This oil has been suggested for inclusion in NAFLD patients' diets, since it reduces insulin resistance and blood triglycerides, inducing a downregulation of lipogenic genes [77]. In a randomised, double-blind clinical trial, the consumption of 20 g/day of olive oil attenuated fatty the liver grade in NAFLD patients [14]. Moreover, in the context of a low caloric diet, the intake of olive oil significantly decreased the levels of alanine aminotransferase and aspartate aminotransferase [78]. Finally, Errazuriz et al. [79], in a randomised trial with prediabetic patients following an isocaloric diet rich in olive oil, observed a decrease in liver fat and an improvement in both the hepatic and total insulin sensitivity.

Dietary Patterns and NAFLD

To discuss the relationship between food groups and NAFLD, another perspective is to analyse the role of diets in NAFLD. This has the advantage that obtained data are based on people's habitual food consumption, thus being more realistic. It can be easily translated into dietetic counselling. Although this task might seem easier to us, it is not so owed to the several different particular diets and dietary quality indices that should be considered: (1) Dietary Energy Density; (2) Dietary Diversity Score; (3) Healthy Eating Index; (4) Healthy Diet indicator; (5) Mean Adequacy Ratio; (6) Diet Quality index and (7) Mediterranean Diet Scale. These indices are important tools to assess the quality of diets and their relationship with various health outcomes.

The Western dietary pattern is a diet with inadequate fruits, vegetables, whole grains, legumes, fish and low-fat dairy products and excessive amounts of refined and processed foods, alcohol, salt, red meats, sugary beverages, snacks, eggs, and butter. It usually implies an excess of calorie consumption. This type of diet is associated with NAFLD independent of physical activity [80]. Besides the role of different foods consumed, an important aspect is that the excess of calories presents a risk factor for NAFLD [81]. In fact, a caloric restriction induces a decrease of several traits related to NAFLD [82].

In the last decade, several studies have analysed the beneficial effects of some dietary patterns on NAFLD. Of particular interest are the Mediterranean Diet and Dietary Approach to Stop Hypertension (DASH). The former was first defined by Ancel Keys in the 60s as a diet low in saturated fat and high in vegetable oils. Ever since, this concept has evolved and varied. Differences in definitions of this diet could limit our knowledge of its health benefits. A good general definition of this diet understands the high intake of extra virgin olive oil, vegetables including leafy greens, fruits, cereals, nuts and pulses/legumes; moderate intakes of fish and other meat, dairy products and red wine and low intakes of eggs and sweets. The adherence to this diet is measured by a score [83]. At present, several studies (observational studies and short-term trials) have demonstrated this type of diet to be beneficial for NAFLD [84]. Two of these studies have showed that the Mediterranean diet score was negatively correlated with serum alanine aminotransferase and the severity of steatosis [85, 86]. Remaining studies indicated similar results. However, it is important to note that longer-term trials with more patients with histological outcomes are required to strengthen the concept of the beneficial effects of the Mediterranean diet on NAFLD. An important aspect of this diet is that it has the potential to improve NAFLD without changes in body weight, which is a big obstacle in lifestyle changes.

The DASH diet originated in the 90s and is rich in in fruits, vegetables, whole grains, fish, poultry, nuts, legumes, low-fat dairy products, reduced sodium, added sugars, as well as saturated and total fat. DASH emphasises on the consumption of minimally processed and fresh food. It designed to regulate blood pressure, but has been found to have beneficial effects in metabolic disorders, such as NAFLD, as found in a case-control study [87] and a randomised clinical trial [88].

Both these diets probably exert their beneficial effects owed to their macro- and micronutrient components. However, the importance of the diet on NAFLD may be mediated by several mechanisms; diet-induced modifications in gut microbiota could be an important a factor. Changes in the gut microbiota composition increases gut permeability and, consequently, the translocation of bacteria and their products. This induces an endotoxemia reported to contribute to liver inflammation in NASH patients [89]. A recent study established a relationship between gut microbiota and NAFLD [90]. They described the gut microbial profile of children with NAFLD. In this regard, we need additional studies investigating the mediation of the NAFLD and microbiota relationship via diets.

Nutritional Geometry and NAFLD

A problem that occurs when studying the role of nutrition/foods/diets on metabolic diseases is that the involvement of particular nutritional parameters in these diseases' traits are in some cases unclear. Moreover, in metabolic diseases, the role of the biology-environment relationship is very complex. Additionally, we eat foods composed of a mixture of nutrients. These are combined into meals and the meals

constitute the diets and dietary patterns influencing health. It is well known that diets are more than the sum of their components. All these aspects have made us to consider that the single-nutrient model paradigm (where, only a cause-effect relationship occurs between a particular disease and a specific nutrient) is not useful to approach metabolic diseases. Emphasising specific nutrients does not take into consideration that food components interact in a high degree of complexity to give rise to emergent properties of diets that escape the level of an individual chemical compound. For example, changing the concentration of one component in the diet can alter everything. We thus have to consider the large number and huge variety of nutrients, foods, diets and dietary patterns that constitute human nutrition. Thus, as outlined in the previous section, we should change the level-of-focus problem and give priority to foods, diets and dietary patterns. It is important to find a methodology that accounts for the interactions among nutrients within foods and diets. The methodology should be able to define and quantify the effects of different diets on multiple health outcomes.

To overcome this problem, Raubenheimer et al. [91] developed the concept of "nutritional ecology". Their framework is that health and disease arise from the interaction between living organisms and their environment. Thus, nutritional ecology focuses on the dynamic interface between the organism and its environment from a nutritional point of view. There exists an integration between nutrition, animals and environments. The perspective of this approximation is how nutrients and foods can be combined in a model to understand how food components interact to regulate the properties of diets affecting health. The modelling approach from nutritional ecology is what it is termed as nutritional geometry.

This approximation models the relationship among different levels of nutritional combinatorial hierarchy (nutrients, foods, meals and diets) using the right-angle mixture triangle geometric model [92] (Fig. 4.3). However, the model can use micronutrients, a combination of macro- and micronutrients, bioactive compounds or other food components. Usually, the model represents 3-dimensional macronutrients (fats, carbohydrates and proteins) of foods and how these combine into a meta-mixture: for example, meals and diets [93]. The levels of the hierarchy of meta-mixtures represented in the model can change depending on the question being addressed. Instead of macronutrients, we can choose foods, dishes, daily meals or dietary patterns. For example, we can test for a specific diet with interactive effects of dietary energy, protein, fat and carbohydrate on food intake, cardiometabolic phenotype and longevity [94]. In this study, the authors have used nutritional geometry to understand how the balance and concentration of macronutrients affect the feeding, aging and cardiometabolic health in an animal model. They have quantified in mice the role of macronutrients on food intake, body composition, lifespan, reproduction, cardiometabolic health, the immune system, mitochondrial function, microbioma and nutrient signalling pathways. An important conclusion of studies using nutritional geometry is that the balance of macronutrients affect food, energy intake and various physiological functions in varied manners [95].

Some questions addressed by nutritional geometry are as follows: (1) how does dietary macronutrient balance relate to energy intakes? [96]; (2) how does

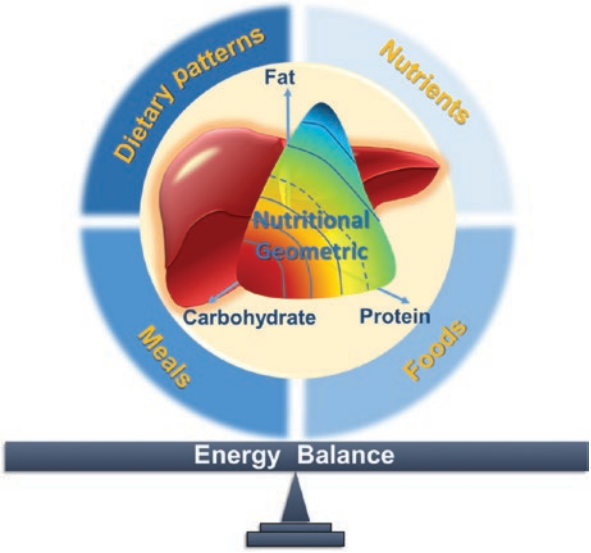

Fig. 4.3 Nutritional geometry in NAFLD. This approach allows to plot foods, meals, diets and dietary patterns together based on their nutrient composition, helping to establish patterns in the links between certain diets and NAFLD

the range of energy intake relate to energy balance? [96]; (3) what is the relationship between dietary macronutrient ratios and total energy intake? [97]; (4) what is the relationship between dietary macronutrients ratios and socioeconomic status? [98] and (5) how does dietary balance influence protein intake? [96]. Another unexplored issue that can be analysed by nutritional geometry is the quality of macronutrients.

Thus, nutritional geometry accommodates multiple diets' components with different animal models and particular health issues. Till date, there is a single study that has used nutritional geometry to evaluate the relationship between diet and NAFLD [94]. This was performed on mice. As indicated by Simpson et al. [99], the authors found that the development of NAFLD at old age increases once carbohydrate intake is over 25 kj/day. When the protein intake exceeds 10 kj/day, the probability of avoiding NAFLD increases. Finally, the highest probability of suffering from severe NAFLD occurs when following low-protein, high fat diets. Moreover, the authors concluded that the ratio and quality of macronutrients could be as important as the diet's energy content.

Conclusions

The relationship between nutrients/food/meals, dietary patterns and NAFLD has been extensively studied over the last 10 years. Research into the role of nutrition in the management of NAFLD patients is a big challenge; this is considerably important as lifestyle modifications consisting of diet, exercise and weight loss have proven to be effective to control the disease. Following calorie-restricted diets over

a long term is associated with an improvement in several features of NAFLD. The specific macronutrient composition of the diet seems to be less important, although this issue remains unclear. What is known is that in the context of hypocaloric diets, both high fat/low carbohydrate or low fat/high carbohydrate intakes do not make any difference, being equally effective in reducing liver lipids. Concerning diets, the Western style diet associates with a greater risk of NAFLD. Contrarily, the Mediterranean diet has shown a significant improvement in steatosis, even in the absence of weight loss. An important problem affecting the research of nutrition in NAFLD is the low progression of the disease. Furthermore, prospective long-term trials with liver biopsies are required to monitor histopathological endpoints. In this context, nutritional geometry approach can be an excellent tool to study relationships between the various aspects of diet, nutrients and liver health. Models can be used in patients to understand the multiple dimensions and relationships between nutritional issues and NAFLD. This would allow for the generation of predictable models to establish personalised nutritional counselling, to prevent and treat NAFLD. The road ahead is thus long, full of illusions and good perspectives for patients.

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Chapter 5
Biomarkers in Pediatric NAFLD

María Rubio-Murillo and Alejandro Rodríguez-Martínez

Introduction

Concepts and Epidemiology

Pediatric non-alcoholic fatty liver disease (NAFLD) is typically defined as hepatic fat fraction (HFF) greater than 5% in the absence of significant alcohol consumption, with no evidence of hepatocellular injury in the form of ballooning of the hepatocytes [1].

Schwimmwer et al. utilizing pediatric autopsies on 742 subjects between the ages of 2 and 19 years revealed a prevalence of NAFLD of 9.6% in north American children [2]. 2.96% showed non-alcoholic steatohepatitis (NASH), but NASH was shown in about 23% of children with NAFLD. The prevalence of NAFLD rose up to 14–83% in obese children [3], meanwhile just 5% of normal weight children had steatosis in that study. Obesity levels have reached epidemic proportions worldwide, with an estimated 1.5 billion adults and 200 million school-age children around the world either overweight or obese [1].

Obesity is the most relevant risk factor NAFLD in children: 40% of children with biopsy-proven NAFLD are obese [4], and just 5% of normal weight children had steatosis in an autopsy study [2]. Along with the recent increase in obesity prevalence, NAFLD has emerged as a medical challenge for pediatricians during the past two decades [3].

From an historical perspective, scientists recognized an association between obesity and fatty liver more than 100 years ago. However, it was not until 1980, when *Ludwing* first used the term of NASH to describe the progressive form of liver

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disease in obese diabetic female patients in the absence of alcohol consumption. Three years later, the first three cases of NAFLD were described in children [5].

NAFLD includes a wide spectrum of diseases ranging from steatosis, or simple lipid accumulation in hepatocytes, to a more advanced injury state called nonalcoholic steatohepatitis (NASH), which is the consolidation of steatosis in the presence of cell injury, inflammation, fibrosis, cirrhosis and hepatocellular carcinomas [3]. The same study of Schwimmwer et al. demonstrated that almost one third of children with NAFLD also had NASH [6]. NAFLD is currently the most common cause of chronic liver disease in children and adolescents in the United States [7]. Epidemiology projections identify this as the most common cause of liver transplantation in children in the near future [8]. On the other hand, the strong association of NAFLD with insulin resistance (IR) and other phenotypic manifestations of metabolic syndrome (MetS), including visceral adiposity, type 2 diabetes, hypertriglyceridemia and arterial hypertension is well established: 51–62% are insulin resistant, and 2.3% have type 2 diabetes mellitus [4, 9].

NAFLD may develop very early in young children, most patients are diagnosed after age 9, but cases reports described children affected as young as 2 years old, and cirrhosis developing as early as 8 years old [4, 10]. There are also significant sex differences in regard to NAFLD. It is more common in boys than girls 2:1. These sex differences implicates estrogens as potentially protective, or indicate that androgens may aggravate NASH [7]. It has been described ethnic disparity in the prevalence of NAFLD with consistent studies. It is more common in Mexican Americans than Caucasian Americans [11]. In some studies, Hispanic children were overrepresented in the NAFLD group [6]. These differences also could be related to several factors including type of diet, exercise choice, socio-economic status and living location. The lowest rate is seen in African Americans despite the high rates of diabetes in this ethnic group [7].

NAFLD in Children vs. Adults

NAFLD in children shares features of adult NAFLD but also shows many different characteristics in terms of prevalence, histology, diagnosis and management [5]. Prevalence in adults ranges from 20 to 30%, both in adults and children has been associated with increased risk in Hispanics. Pediatric NAFLD may be more severe compared to NAFLD identified in adulthood. NAFLD in children is associated with a significantly shorter long-term survival as compared to the expected survival of the general population of the same age and sex. Fifteen percent of children with NAFLD have stage 3 fibrosis or higher at diagnosis. Given that pediatric disease is by definition an early-onset disease, it may represent an aggressive phenotype of the disease. In fact, progression to advanced fibrosis and cirrhosis during childhood is unknown due the lack of prospective studies evaluating children over time [10, 12]. A report published until 13.8-fold higher risk of dying or requiring liver transplantation than the general population of the same age and sex, but NAFLD

with severe NASH may recur in the allograft [13]. In regard to histology, again Schwimmwer et al. published a study analyzing 100 consecutive liver biopsies of children with NASH [14]. This paper suggested two subtypes of histopathology: Type 1 NASH, (resembling an adult-type pattern) was seen in 17% of children and was characterized by steatosis (perivenular, acinar zone 3) with ballooning degeneration and lobular inflammation, with or without perisinusoidal fibrosis and without portal inflammation and Type 2 NASH which is the predominant pattern in children (51%) and it was defined by macrovesicular hepatocellular steatosis (periportal, acinar zone 1) with portal inflammation, with or without portal fibrosis and no or minimal ballooning degeneration. Children with type 2 NASH are more likely to be male, younger, heavier and non-white. Rest of 32% of patients in this study had a pattern of "overlap" [5, 14].

A further distinction could be made between children and adults: the exclusion of other entities that can be processed with hepatic steatosis is more frequent in the pediatric case, mainly, genetic liver diseases. Clues to think this would be: early onset liver disease (e.g., preschool age), evidence of fatty liver in the context of a lean phenotype, significant dyslipidemia or another atypical features. In these cases, do not forget these diseases:

- Lysosomal acid lipase deficiency (cholesteryl ester storage disease): patients with significant hepatosplenomegaly, prepuberal evidence of advanced liver fibrosis or cirrhosis, xanthelasma, or family history of unexplained hepatic dysfunction or early onset cardiovascular disease. These patients tend to have greater elevations of serum LDL compared with patients with NAFLD, and develop premature atherosclerosis. A fulminant infantile form is Wolman disease, characterized by hepatosplenomegaly, hepatic fibrosis, failure of thrive, and adrenal calcifications or insufficiency.
- Abeta/hypobetalipoproteinemia: these disorders are suggested by the findings of low triglycerides and undetectable or low LDL. Abetalipoproteinemia typically presents in childhood with more severe symptoms including steatorrhea, failure to thrive and progressive neurologic complications. It is not common cause of fatty liver in old child.
- Lipodystrophy. This disorder is characterized by abnormal fat distribution in the context of lean body habits, insulin resistance and dyslipidemia.
- Fatty acid oxidation and mitochondrial disorders. Fatty acid oxidation defects (FAOD) are inherited metabolic diseases caused by deficiency of specific enzyme activities or transport proteins involved in the mitochondrial catabolism of fatty acids, leading to tissue accumulation of characteristic fatty acids and L-carnitine derivatives. Affected patients usually present with severe hepatopathy, cardiomyopathy and skeletal myopathy, whereas some patients may suffer acute and/or progressive encephalopathy whose pathogenesis is poorly known [15].

Other disorders to exclude are more common also in adults: viral hepatitis, celiac disease, hypothyroidism, autoimmune hepatitis, Wilson disease or Alpha 1 antitrypsin deficiency.

Pathogenesis

The pathogenesis of NASH is not fully understood. A "two-hit" hypothesis has been described: the first hit involves the accumulation of triglycerides within the hepatocyte. This step is in relation with obesity and IR, but this is not enough to induce progressive liver damage. The second hit leads to hepatocellular injury and inflammation and it involves oxidative stress, lipotoxicity, adipocytokines, alterations in mitochondrial permeability and stellate cell activation [5, 7, 16]. In this way, IR would cause increased influx of free fatty acids (FFAs) to the liver, causing fatty infiltration and the increased levels would induce liver damage via lipid peroxidation, formation of reactive oxygen species (ROS) and mitochondrial disfunction. The result of this inflammatory reaction is the apoptosis and fibrosis originating steatohepatitis. In conclusion, nature of NASH progression starts with lipid metabolism and accumulation, and leads to oxidative stress, inflammation and apoptosis [1, 5] (Fig. 5.1).

Actually, the traditional "two-hit" hypothesis has been replaced by a "multiple parallel hits hypothesis", in which many diverse processes act in parallel, considering

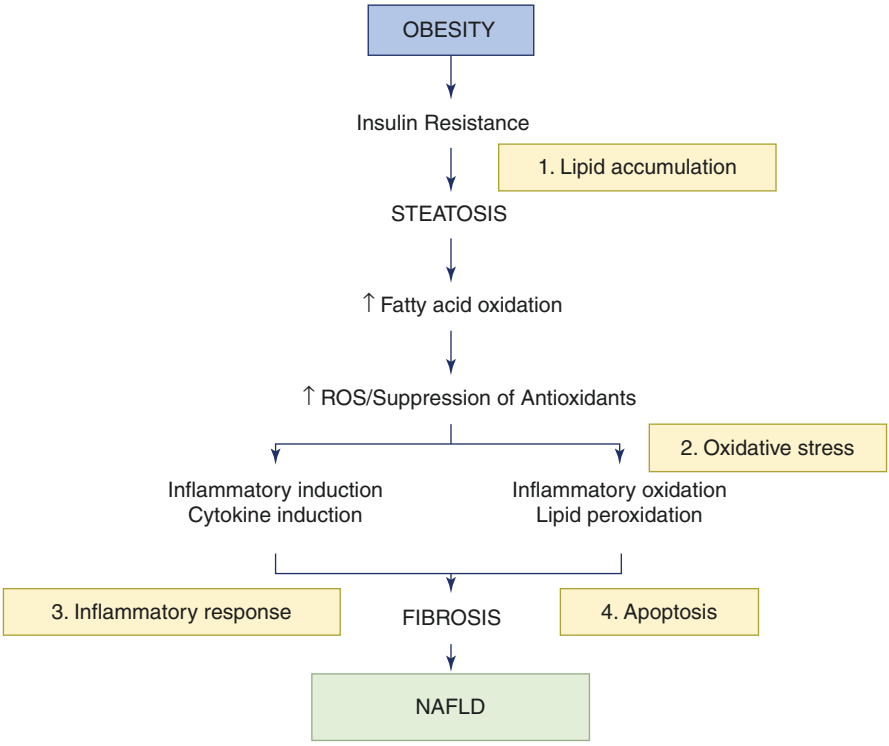

Fig. 5.1 Primary mechanisms involved in the development of NAFLD. Reactive oxygen species (ROS)

the pathogenesis more complex than two hits [17]. This hypothesis emphasizes the importance of the gut-fat-liver axis and its activation. An increase in gut derived endotoxins due to gut permeability would play a crucial role in the development and progression of NAFLD [5].

NAFLD aggregates in families. Loomba et al. showed that early-onset paternal obesity increased the risk of suspected NAFLD in offspring [18] and Schwimmer et al. reported that 17% siblings and 37% of parents of obese children without NAFLD had fatty liver by magnetic resonance spectroscopy (MRS) compared to 59% of siblings and 78% of parents of children with biopsy-confirmed NAFLD [7].

Both in adults and pediatrics, this disorder is highly heritable in which genetic variations and environment closely interact to determine the disease phenotype and the progression to the more advanced forms of the disease [5]. Human genome-wide association studies (GWAS) in adults have provided important insights into the genomic variation in NAFLD and have identified specific loci contributing to fat accumulation in the liver and NASH development [19]. Recent pediatric studies have provided evidence for the interplay between host genetics and environment in human NAFLD. Santoro et al. demonstrated that the consumption of a diet with a high n-6 to n-3 polyunsaturated fatty acids (PUFA) ratio resulted in higher hepatic fat fractions as assessed by liver magnetic resonance imaging (MRI). However, this effect was seen only in children with a genetic polymorphism in the patatin-like phospholipase domain-containing 3 (PNPLA3) gene, which has been identified as the strongest modifier of NAFLD and NASH pathogenesis in most of the GWAS performed to date [20]. Another study analyzed the crosstalk between diet and genes with regards to liver cell death and liver injury [21]. This study demonstrated that the association between hepatic fat accumulation and liver cell death may be in part dependent on ethnicity. In this study of over 220 obese children with the same degree of hepatic fat content, obese African American youths showed lower levels of liver cell death than their Caucasian and Hispanic controls, independent of the degree of insulin resistance.

Further studies from genes will contribute to a better understanding of the NAFLD susceptibility and differences based upon ethnicities and gene-environment interactions.

Diagnosis Challenge

Early diagnosis is necessary in order to avoid fibrosis progression. The presence and severity of fibrosis in these patients are important prognostic factors for the risk of disease progression to cirrhosis. Screening for NAFLD should be performed in all children with obesity (body mass index (BMI) $\geq$ 95 percentile), and for those who overweight (BMI > 85 percentile) if other risk factors are present (e.g., signs of insulin resistance (IR) or a family history of NAFLD). Screening should be initiated between 9 and 11 years old [10].

The gold standard for staging liver fibrosis is a liver biopsy. However, the biopsy is an expensive and invasive procedure, and it has some inherent risks. Because of

these limitations, it is interesting and desirable to develop useful biomarkers that could better discriminate between simple fatty liver and NAFLD complicated by NASH for no invasive diagnosis of liver fibrosis [6]. Ideal biomarker should be minimally invasive, readily available, accurate and cost effective [1, 4].

The object of this chapter is the updating of such biomarkers in pediatric practice. Multiple biomarkers have been studied in adult patient in an attempt to diagnose NASH non-invasively. However, the available data on biomarkers in children are limited and require further validation before integration into clinical practice.

In the same way, in adults, other predictive models which combine routinely assessed clinical variables with laboratory tests and biomarkers have been proposed to make the diagnosis of NASH [5]. It would be very interesting to find a model like this or a panel that includes the best biomarkers in children to apply in areas where obesity and its comorbidities are salient. There are some studies in this respect, although unfortunately need further external validation.

Biomarkers

As we have already mentioned, the available data on biomarkers in children are limited. Multiple biomarkers have been studied, but the reports are isolated studies with a very limited number of patients. There is a lack of extensive reviews about the same biomarker and reproducible studies which provide validity to an isolated marker.

Thus, despite a recent increase in research efforts to identify biomarkers for NAFLD and NASH, currently the only marker which appears in Clinical Practice Guideline in pediatrics is the quantification of the transaminases, and yet, this marker has been evidenced to have many limitations. In this chapter we will review transaminases in first place as a clinical marker of NAFLD in pediatrics and then we will stop at the most promising biomarkers which have demonstrated some relationship with NAFLD in pediatrics on the basis of their contribution to physiopathology of the entity.

The American Academy of Pediatrics recommends that alanine aminotransferase (ALT) and aspartate aminotransferase (AST) level measurements should be performed to screen NAFLD in overweight children (BMI between the 85th and 94th percentile for age and sex) with risk factor and in obese children (BMI $\geq$ 95th percentile for age and sex) even in the absence of risk factors [21]. ALT, is a serum marker of liver damage, is inexpensive, universally available blood test, is minimally invasive and has an acceptable sensitivity. In the last NASPGHAN Guideline for the Diagnosis and Treatment of NAFLD in children [12], they proposed cut-off values for an elevated ALT (>26 U/L in boys, >22 U/L in girls) as screen. For the diagnosis of NAFLD, the use of two times the sex-specific ALT (ALT >50 for boys and >44 for girls) in overweight and obese children age 10 years or older has a sensitivity of 88% and a specificity of 26%. In any case, the interpretation of levels of transaminases is ambiguous and has limitations: ALT is not sensitive enough to predict with certainty the presence of NASH or the stage of fibrosis [12, 21–23].

However, NASH is more common in children with ALT ≥80 U/L compared to those with ALT <80 U/L (41% compared to 21%, respectively) [12].

On the other hand, the sensitivity to change (discriminant validity) is not well documented, neither in pediatric NAFLD [24]. Some studies have evaluated the utility of ALT as a predictor of histologic progression versus improvement of pediatric NAFLD over time. Vuppalanchi el al [25]. showed in an analysis of ALT in the clinical trial of treatment of nonalcoholic fatty liver disease in children (TONIC) that for every decrease of 10 U/L, the relative odds of histologic improvement and resolution of NASH were 1.31 and 1.26 respectively. Later, Arsik et al. published secondary analysis using TONIC trial data and they concluded that a single ALT measure will not accurately predict NASH or fibrosis, but rather they show that in patients with both histologic disease and an elevated ALT >60 at baseline, the change in mean of ALT over time is reasonable biomarker of improved histology [24].

For the rest of transaminases, AST and gamma-glutamyl transferase (GGT) have not been independently tested as screening tools for NAFLD in children. In the context of elevated ALT, higher AST and higher GGT are associated with worse histology. Elevated AST or GGT in the context of normal ALT may, however, represent a condition other than NAFLD [12].

The growing understanding of the pathophysiological mechanisms involved in disease progression in NAFLD has allowed the testing of several mechanism-based biological markers targeted at specific pathways involved in liver damage and disease progression to NASH. In this respect, attending the pathophysiology, we find biomarkers of fat accumulation, biomarkers of oxidative stress, biomarkers of inflammation and biomarkers of apoptosis. Then we will develop such markers have more relevance in pediatrics.

Biomarkers of Fat Accumulation

Biomarkers for lipid and carbohydrate metabolism and accumulation have been associated with fatty liver. The primary form of fat in the liver is triglycerides. Based on both human and animal studies, expansion of the intrahepatic pool of FFAs is the reason for triglyceride accumulation in NAFLD. In these patients, the pathways which lead to FFAs efflux are usually functioning at a higher level, indicating that the critical step leading to fat accumulation is related to excessive inflow of FFA [1]. Studies have shown that up to 75% of the FFA pool is derived from excess adipose tissue lipolysis. In adults, fibroblast growth factor-21 (FGF-21), apolipoprotein B (Apo-B), FFA, IR, fatty acid transporter 5 (FATP5) and IR are some of the markers reported. In pediatrics the biomarkers of fat accumulation more significant are *IR*, as clinical variable which reflect fat accumulation and *cathepsin D (CatD) levels*.

– *IR*. *IR* is the primary metabolic disorder associated with obesity and is defined as a diminished ability of insulin to stimulate glucose uptake by skeletal muscle and adipose tissue in addition to reducing insulin's ability to suppress hepatic glucose production and output [26]. *IR* is a common pathway for the development

of glucose metabolism disorders, dyslipidemias and high blood pressure, all of which are components of the MetS. In obese patients, insulin sensitivity is reduced. An Egyptian group proposed to determine the association between *IR* and both NAFLD and MetS. The study included 76 overweight/obese children. *IR* was detected in 43.4% and 34.2% by using the quantitative insulin-sensitivity check index (QUICKI) and the homeostasis model assessment method (HOMA-IR), respectively. NAFLD was detected in 45.5% among those performing liver biopsy. Cases with NAFLD had more frequent *IR* than children without NAFLD [27]. This report is not the only one in this line. Other studies in obese children and adolescents reported that NAFLD cases had more prevalent *IR* [28, 29]. These results suggest that markers of insulin sensitivity could be useful screening parameters for NAFLD.

– *CatD*. Cathepsins, the main class of lysosomal proteases, have been described to have an early role in inflammation. Walenbergh et al. reported the role of plasma *CatD* levels to predict pediatric hepatic inflammation. They are based on numerous studies which have shown that lysosomal cholesterol accumulation inside macrophages induces disturbances in lysosomal trafficking, and it is an event that occurs during inflammation and has been detected in NASH as well as in atherosclerosis [30]. The exact mechanisms that lead to the secretion of the lysosomal content in plasma are not yet known. In the report, they enrolled 96 children with liver biopsies with NAFLD according to the criteria of Kleiner [31] (NAFLD activity score) and the Brunt's criteria [32]. They reported that levels of plasma *CatD* are decreased at early stages of NASH compared with steatotic subjects. They found that *CatD* levels were gradually reduced and corresponded with increasing severity of liver inflammation, steatosis, hepatocellular ballooning, and NAFLD activity score. *CatD* levels showed better correlation than ALT and CK-18 with a high diagnostic accuracy for the differentiation between steatosis and hepatic inflammation. With this conclusion, authors proposed this biomarker could be used as a promising non-invasive marker in NASH [33]. Nevertheless, we have to take into account the limitations of this study: children with normal levels of aminotransferases are not included, because obtaining liver biopsies from those children is not a common practice. Consequently, the level of plasma *CatD* under healthy conditions is not known. More studies in this way have not been published, so whether *catD* can be used in clinical practice should be validated in additional larger well-defined NAFLD cohorts in children and adults.

Proinflammatory and Stress Oxidative Biomarkers

While fat accumulation is the common denominator of all forms of NAFLD, an important distinction to make is that fatty infiltration alone does not lead to NASH and cirrhosis. Excess lipid accumulation results in toxic effects on hepatocytes: oxidative stress triggers inflammation and wound healing that eventually cause fibrosis.

Lipid accumulation and lipotoxicity can contribute to inflammatory changes that result in NASH, whereas antioxidant activity within the liver is believed to protect hepatocytes from oxidative stress. The overproduction of lipid peroxidation products by several oxidation pathways as FFA oxidation via peroxisomal β-oxidation and the microsomal ω-oxidation, can provide quantifiable biomarkers [1, 9, 23]. In addition to this, malfunction in protein associated with metal homeostasis, such as ferritin, ceruloplasmin (Cp), could exacerbate oxidative stress. *Bilirubin*, *dysfunctional HDL*, *8-isprostone levels*, *Cp*, *ferritin*, are example of biomarkers of stress oxidative. In children *bilirubin*, *Cp* and *ferritin* have been reported in some studies.

In relation to proinflammatory biomarkers, cytokine imbalance has been demonstrated in both adults and children with NASH. Chronic systemic inflammation plays a critical role in the development and progression of NAFLD from simple steatosis. The mechanisms by which inflammation leads to NASH is still unclear. NAFLD is considered to be the hepatic manifestation MetS [34]. It is believed that IR and a chronic low-grade inflammation are through to lead to the development of NAFLD in genetically predisposed individuals [1]. Given the complicated nature of NASH progression many markers are involved in multiple steps of the process and cannot be delineated so clearly. Several inflammatory markers, which also happen to be markers of oxidative stress and apoptosis, have been proposed as markers of NAFLD and NASH: *pentraxin 3 (PTX3)*, *increased leptin*, *elevated IL-6*, *TNF-α*, *decreased adiponectin*, *acid hyaluronic (HA)*.

– *Bilirubin*. *Bilirubin* is a potent endogenous antioxidant with cytoprotective properties. The antioxidant effect of bilirubin in inhibiting lipid peroxidation may even exceed that vitamin E, a strong antioxidant [35]. Higher levels of *bilirubin* have been shown to be inversely associated with IR and MetS in children and adolescents [36]. In 2013, a report published a cohort of 302 children with biopsy-proven NAFLD. The mean total *bilirubin* was significantly lower in the NASH group compared with the non-NASH group. Higher total bilirubin levels were negatively correlated with the presence of steatosis and the NAFLD activity score ($p < 0.05$). They concluded that in children with NAFLD there is an inverse relation between serum *bilirubin* levels and the presence of NASH on biopsy and this may be secondary to the oxidant effect of *bilirubin*. In adults have been published some studies in this respect, showing a significant relation between *bilirubin* levels and NAFLD severity [37]. These result in children give us an opportunity to identify those patients who may be at a higher risk for developing a NASH and may require even closer follow-up [17]. The main weakness of this biomarker is the few reports in children, so further studies are needed to confirm this association.
– *Ceruloplamin*. Nobili et al. suggest ceruloplasmin as a biomarker in NAFLD. They are based on studies of animal models with NAFLD and humans in which have been pointed out condition of copper deficiency associated with lipid accumulation and oxidative stress. They calculated an index of the activity of the antioxidant Cp-transferrin system in 100 children with biopsy-proven NAFLD. Pediatric patients were grouped by non-alcoholic fatty liver disease score (NAS) ≥ 5 and < 5.

The main result of this study is that *Cp* decreases seem to discriminate children with more severe NAFLD. Specifically, a cut-off of 28.6 mg/dL distinguished NAS ≥ 5 from NAS < 5 with a specificity of 92% and a sensitivity of 76%, pointing out its potential as a noninvasive and supportive marker of the disease. *Cp* was also associated with steatosis; however, the accuracy of this prediction was much lower than that with NASH, ballooning and inflammation and not enough to be used for practical purposes [9]. Diverse hypotheses suggest that *Cp* variations in NAFLD can be reflection of liver dysfunction, which is also the case in Wilson disease and aceruloplasminemia which share some signs with NAFLD. The lower serum *Cp* levels may resemble a lower intrahepatic *Cp* content secondary to liver dysfunction, reflecting higher susceptibility to oxidative stress at the hepatocyte level, in terms of peroxidation or accumulation of lipids, which may result in ballooning formation in NASH. As conclusion, *Cp* detection may serve as an additional noninvasive test for the screening of children with suspected NAFLD at liver function tests and ultrasound. Limitations of this study including the need for patient selection on the basis of NAS score, the lack of data on healthy controls, and the small number of cases analyzed. Another limitation above the copper as biomarker is the few studies. Only *Nobili* has reported results in children and in adult studies are few too [38, 39].

– *Ferritin*. Increased *ferritin* but normal transferrin saturation is frequently found in patients with hepatic steatosis. The simultaneous disorder of iron and glucose and/or lipid metabolism, in most of the cases associated with *IR*, is responsible for persistent hyperferritinemia and identifies patients at risk for NASH [35]. In NAFLD, increased *ferritin* levels are considered an expression of metabolic syndrome and of hepatic damage, because of inflammatory cytokine activation. Hepatic iron accumulation produces inflammatory cytokines, and they induce hepatic fibrosis [40, 41]. There is only one report which studied serum *ferritin* to pediatric NAFLD patients based on previous adult studies [40, 42]. The group of Na et al. analyzed the correlation between serum *ferritin* and laboratory values including NAFLD severity markers in obese group and overweight group of 46 children. The results of the comparison showed that serum *ferritin* was related with the severity of NAFLD. Thus, authors proposed *ferritin* as possible biomarker of NAFLD in children. However, they did not compare the liver biopsy tissue of each patient with their serum *ferritin* level and they pointed out that there are more factors that can affect serum *ferritin* levels. Unfortunately, not there are more publications on this marker in children, so the evidence for the application is low.
– *PTX3*. *PTX3* is a prototypic member of the long chain pentraxin family and a marker of the acute phase inflammatory response [34]. *PTX3* is produced by variety of tissues and cells, in particular by innate immune cells, in response to proinflammatory signals. It was suggested that plasma *PTX3* levels may differentiate NASH patients from other subjects, and that higher plasma *PTX3* levels are associated with severe stages of hepatic fibrosis [43]. Hamza et al. evaluated the clinical utility of serum fragment levels in the diagnosis of NASH and the assessment of its severity in obese children with suspected NAFLD [44]. They

compared 50 obese children to 25 matched controls and showed than *PTX3* was higher in obese cases than controls. Eighty percent of the cases had NAFLD with progressive increases in *PTX3* levels as the severity of fatty liver increased. A cutoff value of 3.03 U/L differentiated fatty liver from NASH with sensitivity of 89% and specificity of 86%. These findings are supported by other studies which showed that serum *PTX3* levels were significantly higher in NASH cases than in non-NASH cases with simple steatosis and in controls and in cases of stage 3–4 NAFLD than stage 0–2 NAFLD [43]. Thus, *PTX3* levels might be used as biomarker in NAFLD for children but unfortunately there are hardly any more studies about this marker in pediatrics.

– *Leptin*. *Leptin* is the most important adipokine produced by adipose tissue. These proteins coordinate insulin homeostasis, immunity, and obesity related inflammation [45]. So, that adipokine might be involved both in the first hit as in the second, according to the classical hypothesis [46]. Fitzpatrick et al. evaluated also the leptin compared with CK18 in children. Leptin could distinguish <F2 from ≥F2; 28.9 ng/mL versus 70.1 ng/mL (P = 0.037) but did not meet statistical significance as a predictor of either NAS score or fibrosis stage [47]. For Sayin et al. serum leptin levels were significantly higher in obese adolescents than in healthy group, but there were not difference in patients with, and without, NAFLD [45]. Boyraz et al. did found that leptin levels in obese children with NAFLD were higher than those in the control group [48]. Really the role of the leptin in fatty liver disease is controversial with ambiguous results in different published studies [45]. These findings require further investigation in extensive studies to support this hypothesis.
– Elevated *interleukins*. The role of *IL-6* in liver pathology is very complex, and its participation in the development of NAFLD remains unclear. *IL-6* activates several cells, such as immune cells, hepatocytes, hematopoietic stem cells, and osteoclasts. Furthermore, *IL-6* has a wide range of biological functions, including induction of inflammation and oncogenesis, regulation of immune response, and support of hematopoiesis. *IL-6* was initially considered as a hepatoprotector in liver steatosis, capable of reducing oxidative stress and preventing mitochondrial dysfunction [49]. In humans with NASH, a positive correlation between *IL-6* expression in hepatocytes and the severity of NAFLD was observed [50]. In children we do not find strong evidence of association. There are very few reported studies. Assunçao et al. published a report with the objective to describe the behavior of proinflammatory cytokines in obese children and adolescents, with and without non-alcoholic fatty liver disease. They did not find significant correlation between the severely obese and *IL-6*, showing however, a tendency for association [51]. A previous study by Perito et al. investigated the relationship between plasma cytokine levels and features of NAFLD histology. Cytokines were measured from plasma obtained at enrollment in pediatric participants (235 subjects) in NASH Clinical Research Network studies with liver biopsy-proven NAFLD. They concluded that *IL-8* increased with steatosis and fibrosis severity, *sIL-2rα* increased with fibrosis severity and portal inflammation, *IL-7* decreased with portal inflammation and fibrosis severity but they did not find association

between *IL6* and hepatic fibrosis [52]. Neither *Fitzpatrick* could demonstrate relationship between *IL6* and NAFLD in 40 children with liver biopsy-proven [46]. More targeted analysis is needed to identify the role of these markers in NAFLD in children and to evaluate their potential as non-invasive discriminators of disease severity.

– *TNF-α*. *TNF-α* plays an important role IR, the pathognomonic feature of MetS, through inhibiting the tyrosine kinase activity of the insulin receptor [34]. Strong evidence supports a key role *TNF-α* as proinflammatory cytokine in the pathogenesis of NASH [53]. This cytokine mediates in liver injury given its ability to induce inflammation and apoptosis in hepatocytes under conditions of oxidative stress. There are some reports about this topic in pediatrics. A study tested the power of *TNF-α* in predicting the degree of liver involvement in children with NAFLD [7]. They measured serum levels of *TNF-α* and computed NAS ≥5 as diagnosis of NASH in 72 biopsy-proven NAFLD cases. A value of *TNF-α* of 7.9 pg/mL or more has a sensitivity of 82% and a specificity of 96%. This report also proved association with levels of leptin as biomarker but obtaining less sensitivity (54%) and specificity (76%). The sample size is a factor bound to extrapolate the results to the pediatric population and more studies about this topic are needed.
– *Adiponectin*. *Adiponectin* is an important adipocyte-secreted cytokine, adipocytokine, that has anti-inflammatory properties including modulation of inflammatory cells. The inverse association of serum *adiponectin* level with BMI suggests that obesity is an adiponectin-deficient state [54]. In adults, *adiponectin* levels are associated with increased hepatic fat and are decreased in patients with NASH [55]. Vos et al. evaluated the relative concentrations of cytokines in pediatric NAFLD in 30 children. They compared normal-weight children, with and overweight without elevated ALT children and overweight with elevated ALT children (presumed NAFLD). In the obese group and those in the NAFLD group had significantly lower serum *adiponectin* levels ($p < 0.04$). They found that *adiponectin* was the most important factor related to elevated ALT [55], and there was no difference with other proinflammatory cytokines levels. They concluded that *adiponectin* may play a more proximal role than dysregulation of circulating pro-inflammatory cytokines in the mechanisms leading to NAFLD in children. The main limitation in this study is that the group of subjects was very small. Further reports published similar results as the group of Boyraz et al. which concluded that *adiponectin* levels in obese children with NAFLD were lower than those in controls and in obese children with hepatosteatosis [48]. In this group of patients, biopsy had not been performed, so it can be considered an assumed NAFLD. These results were consistent with previous studies in children [56, 57]. In any case, multicenter validation study should be undertaken to evaluate the usefulness of these markers in clinical practice.
– *HA*. *HA* is a glycosaminoglycan synthesized by extracellular matrix (ECM)-producing cells, including activated hepatic stellate cells, and is one of the best predictors of liver fibrosis in adults [58]. *HA* is defined as "direct marker" because this marker is directly linked to modifications in ECM turnover during fibrogen-

esis [59]. Lately, Nobili et al. evaluated the association of *HA* with liver fibrosis in 100 consecutive children with biopsy-proven NAFLD. In all, 65% of the children had liver fibrosis. They found that values of *HA* ≥ 1200 ng/mL made the absence of fibrosis (F0) unlikely, whereas values of *HA* ≥ 2100 ng/mL made F2, F3, or F4 fibrosis likely [3, 60]. This study is the first to show that serum *HA* is a predictor of the degree of hepatic fibrosis in a pediatric population with NAFLD. Lebensztejn et al. published 1 year later findings in agreement with the data reported by *Nobili* [61]. They determined *HA* and *cytokeratin-18 (CK18)* in 52 children with biopsy-proven NAFLD. The levels of *HA* and *CK18* were significantly higher in children with biopsy compared to children without fibrosis. The combination of both markers was superior (AUC = 0.73) than separately. More research should be carried out in order to confirm this association and its utility as a predictor.

Apoptosis Biomarkers

Apoptosis, or programmed cell death, has emerged as an important mechanism in disease progression of NASH. Apoptosis is a highly organized process than can happen by extrinsic via or intrinsic pathway. Both pathways can lead to the activation of caspases, which cleave intracellular substrate, including *CK18*, which is the major intermediate filament protein in hepatocytes. In the same group of apoptosis include markers of extracellular matrix turnover like *HA*. Another apoptosis biomarker studied in children are: *soluble Fas (sFAs)* and its ligand *FasL* and enhanced *liver fibrosis (ELF)*.

– *CK18* is a liver-specific cytoskeletal protein that is cleaved by caspases during hepatocyte apoptosis, releasing fragments that are detectable in serum samples. CK18 has been validated as a marker of NASH in many studies in adults and been shown to be able to distinguish steatohepatitis from hepatic steatosis [62, 63]. Two recent studies with children support this theory. One study included 45 children with biopsy-proven NAFLD and demonstrated that the medium value of *CK18* fragments in children with NASH was significantly higher than those with hepatic steatosis. A cut-off value of 207 U/L gave a sensitivity of 84% and a specificity of 88% [47]. The second study included 201 children, 140 with NASH on liver biopsy. They showed than CK18 levels were significantly higher in patients with NASH compared to those without NASH. For every 10 U/L increase in CK18 levels, the likelihood of having NASH increased by 70% after adjusting for multiple confounders. A cut-off value of 233 U/L gave a sensitivity of 85% and a specificity of 86.9% [64].

In the same line, Vos et al. [6] and Sodhi et al. [1] reported previous studies, but with the limitation that children do not routinely undergo liver biopsy, so they did not directly assess the relation between plasma *CK18* levels and liver biopsy findings. Sodhi et al. showed a correlation between obesity, *IR* and *CK18*. In the obese

and obese with *IR* patients, levels of *CK18* were significantly increased when compared to the control ($p < 0.02$), and compared to obese without *IR* patients. Liver biopsies were not conducted in any cases basing the results on the strong relationship between obesity, *IR*, and NASH.

At present, *CK18* is not considered a valid marker yet in the recent guideline for children [12]. Anyway, it is the most studied biomarker, and just like in adults, *CK18* may become one of the most promising noninvasive tests for diagnosis and managing NASH in children.

- *Fas/FasL complex*. The Fas*/FasL complex* is a key of the extrinsic pathway of the hepatocyte apoptotic cascade. This relation has been proved in adults [65] and recently, the same group of investigation has proved this relation in children. Alkhouri et al. evaluated the plasma levels of *sFas* and *sFasL* in 117 children with biopsy-proven NAFLD. Both *sFas* y *sFasL* were significantly elevated in children with NASH compared to those without NASH. sFasL was found to be better predictor for NASH and had stronger correlation with the histological features of NAFLD than *sFas* [66]. This finding opens a promising line of investigation, anyway more studies are needed to validate this report.
- *ELF test*. *ELF test* uses a combination of three extracellular matrix components: *HA*, amino terminal propeptide of type III collagen (PIIINP), and inhibitor of metalloproteinase 1 (TIMP-1). This group of markers reflects alterations of hepatic extracellular matrix (ECM) metabolism and inflammatory activity within the liver [60]. Consequently, it seems to be the most appropriate for detecting fibrosis in children and adolescents. *ELF test* has been validated for staging liver fibrosis in adult patients with chronic liver diseases, including NAFLD [58]. Nobili et al. investigated the performance of the *ELF test* in the assessment of liver fibrosis in children and adolescents with biopsy-proven NAFLD [67]. In pediatric patients, the *ELF test* predicted liver fibrosis stage with a high degree of sensitivity and specify, and is superior to those reported in adults. A reason that may explain these better results could be the effects of borderline comorbidities and the aging process on extrahepatic extracellular matrix turnover. Organ fibrogenesis are less likely in children and adolescents, particularly, the model of fibrogenesis in pediatric NAFLD is different from adults, as we commented previously, and finally in children there is more efficient degradation and remodeling of scar tissue [68].

Predictive Scores

NASH scores or panels are tools which combine the routine assessment of clinical variables with laboratory tests and biomarkers. Development of such scores may facilitate both identification of specific biomarkers as well as novel therapeutic strategies for pediatric NAFLD [22]. Predictive scores have been also studied in adult patients, but, unfortunately, most of them need further external validation [5]. The same happens for children.

Yang et al. in a comparative study extrapolated scores of adults in pediatrics. They reported that only AST/platelet radio index (APRI) and FIB4 exhibited statistically significant differences between patients with mild fibrosis and those with severe among noninvasive scores published for adults [69].

The first noninvasive score of liver fibrosis designed specifically for the pediatric population was developed in an Italian cohort by Nobili et al. in 2009 and is called the pediatric NAFLD fibrosis index (PNFI) which varies between 0 and 10, and includes three very simple clinical measures: age, waist circumference and triglycerides (TG) [70]. They found that a PNFI value of ≥9 could be used to rule in the presence of any fibrosis and a value of <3 could rule out fibrosis. However, the PNFI was not different between those with and without significant fibrosis, due to the fact that majority of children in the original PNFI cohort had only mild fibrosis (stage 1) and none had cirrhosis, they could not develop a predictor of advanced fibrosis [3, 69].

The group of Alkhouri et al. [66] has developed a prediction model for NASH called the "NASH apoptosis score". It consists in a multivariable logistic regression analysis to predict NASH using available clinical variables: *sFas levels*, *transferrin*, *ferritin*, *TG*, and *age* with an area under the roc curve (AUC) of 0.78. They propose a diagnostic algorithm presented on Fig. 5.2. Using this algorithm, the correct diagnosis could have been made without liver biopsy in 78% of patients.

The group of Nobili et al. with Alkouri et al. have proposed several indexes. Firstly, using the same score PFNI but in combination with *ELF test*. *ELF test* uses a combination of three extracellular matrix components: *HA*, amino terminal propeptide of type III collagen (PIIINP), and inhibitor of metalloproteinase 1 (TIMP-1). The combined results from the PNFI and *ELF test* predicted the presence or absence of fibrosis in 86.4% of children with NAFLD [71].

Another predictive score for the same group for advanced fibrosis called the pediatric NAFLD fibrosis score (PNFS). This model includes: ALT, GGT, alkaline phosphatase and platelets and it was studied in a cohort of 242 children with biopsy-proven NAFLD. The AUC was 0.74 which was higher than the AUC for APRI, NAFLD fibrosis score and FIB4-Index. Probably the fact of including a number of platelets could indicate more advanced liver disease and portal hypertension. In this way it is increased the power to separate children with advanced fibrosis. They suggest that children with PNFS values above that cut-off should undergo a liver biopsy

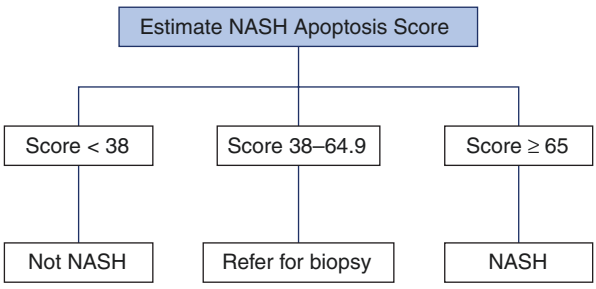

Fig. 5.2 Suggested diagnostic algorithm using the NASH apoptosis score

to confirm the presence of fibrosis. Anyway, PFNS would need external validation before it become a useful tool for routine use [5, 72].

The last score published for the same group of authors, is a score which combines biochemical markers and imaging studies in a cohort of children with biopsy-proven NAFLD. They aimed to evaluate the performance of PNFI in combination with transient elastography (TE). TE is a radiological imaging technique based on liver stiffness measurement. If PNFI is ≥8.2, a TE is performed. A TE score less than 8.6 KPa provided a 100% accurate prediction of early liver fibrosis (F0–F1) indicating no need for liver biopsy. TE score ≥ 8.6 KPa predicts significant fibrosis (F2–F3) and therefore the need for biopsy. They concluded that the combined use of PNFI and TE could predict the presence or absence of clinically significant fibrosis in 98% of children with NAFLD, and this algorithm could be used by clinicians for the early detection of fibrosis [73]. But neither PNFI nor the TE can distinguish between simple steatosis and NASH and their use should be limited to patients with high suspicions of NAFLD. Additional studies are needed to externally cross validate these findings.

A genetic risk score has been published in a study that combined multiple single-nucleotide polymorphisms (SNPs) known to be associated with NAFLD severity resulted in fair accuracy for predicting NASH, with AUC of 0.75, and the accuracy was improved by adding clinical variables, such as age and AST level (AUC 0.80) [21].

Conclusion

A lot of markers of liver injury in children has been studied and published. We have reviewed several of them but none of the markers is yet able to match the precision of tissue sampling. Some may serve as promising non-invasive markers for detecting NAFLD and NASH. The main limitations of these studies are small sample size and the lack of external validation or reproducibility. Currently there is insufficient evidence to support the routine use of any specific biomarkers for assessment of NAFLD and NASH. It seems that every time we are closer to find an ideal biomarker. Or better yet, a panel which meets the most sensitive and easy biomarker to use in clinical practice in order to get a simple tool for the early diagnosis and monitoring of this entity.

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Chapter 6
Biochemical Biomarkers of NAFLD/NASH

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Introduction

The epidemiological burden of nonalcoholic fatty liver disease (NAFLD), the hepatic phenotype of metabolic syndrome, is dramatically increasing over the last years, with incidence rates estimated from 18 to 50 cases per 1000 person-years and a prevalence in general population ranging from about 13% in Africa to more than 25% in Western world [1]. The main reasons of the growing burden associated to NAFLD are represented by the global epidemics of type 2 diabetes and obesity and it is not surprising that the prevalence of NAFLD is higher in at risk populations, like in patients older than 50 years and affected by features of metabolic syndrome [2]. Nonalcoholic steatohepatitis (NASH) is the progressive form of NAFLD and the estimates of its prevalence in general population (ranging from 1.5 to 6.4%) are less accurate, considering that liver biopsy still remain the gold standard for the diagnosis of NASH [1, 3] and for the quantification of steatosis, inflammation and fibrosis.

Today, NAFLD is the new most common cause of chronic liver disease [1], a significant risk factor for hepatocellular carcinoma (HCC) [4] and one of the leading indications of liver transplantation [5]. In addition to liver-related events, patients with NAFLD are at higher risk of extrahepatic morbidity and mortality, especially cardiovascular diseases and extrahepatic cancer, that represent respectively the first and the second causes of death [6]. Liver fibrosis stage has showed to be a significant predictor of hepatic and extrahepatic prognosis of NAFLD patients, as demonstrated in long-term natural history studies [7, 8].

The correct identification of NAFLD patients at high risk for liver disease severity is crucial for clinical decision making, for the stratification of the prognosis and

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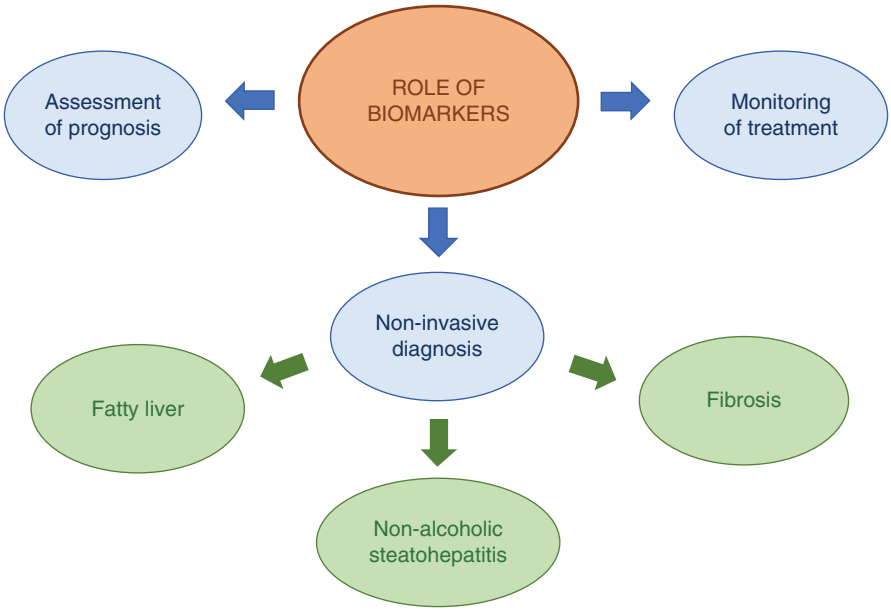

Fig. 6.1 Role of biomarkers

for the management of therapeutic approaches. Several non-invasive biomarkers have been proposed and validated to be used in clinical practice. However, they are useful mainly in population studies, but not for single patients, generating large areas of diagnostic uncertainty [9]. In this chapter, we will focus on the role of non-invasive biomarkers (Fig. 6.1) in: (1) the detection of NAFLD and NASH; (2) the identification and the stratification of patients with severe liver disease, at risk for progression toward hepatic and extrahepatic complications; (3) the response to treatments, either consisting in lifestyle correction or pharmacological.

Detection of NALFD

NAFLD is a typically asymptomatic condition and the diagnostic suspicion is often made following the incidental evidence of abnormal liver tests or fatty liver detected by an ultrasound (US) exam performed for unspecific reasons. However, it should be considered that about 80% of patients with NAFLD have normal alanine transaminase (ALT) [10] and that the severity of liver fibrosis seems to be similar between patients with normal and abnormal liver tests, although these latter have a higher inflammatory activity [11]. Furthermore, it is well known that NAFLD is highly prevalent in patients with metabolic syndrome and it should be suspected also in patients with just some of the features of metabolic syndrome [12]. However, an Italian study showed that in 431 patients with histologically-proven NAFLD, about one third was not affected by visceral obesity, identifying a particular subset of NALFD patients, so called “lean” NAFLD

[13]. For these reasons, and considering the uncertainties about diagnostic tests accuracy and their cost-effectiveness, a systematic screening for NAFLD in general population is not recommended to date [14]. By contrast, screening for NAFLD (by liver tests or US) should be performed in patients with persistently abnormal liver enzymes and in high risk individuals (i.e. patients older than 50 years, type 2 diabetes and metabolic syndrome) [3]. The gold standard for the diagnosis and the quantification of fatty liver remains liver biopsy, although it could not to be representative of liver fat, and it is limited by intra- and interobserver observer variability in the definition of pathological features. For these reasons, in general population, several imaging techniques, such as US, controlled attenuation parameter (CAP), and spectroscopy magnetic resonance (MR) have been studied for the diagnosis of fatty liver (not the topic of this chapter).

The main scores and algorithms studied for the detection of fatty liver include the Fatty Liver Index (FLI) [15, 16], the Kotronen score [17], the Lipid Accumulation Product (LAP) [16, 18], the Korean study score [19], the Hepatic Steatosis Index (HSI) [20], and the SteatoTest [21, 22] (Table 6.1). FLI is an easy to calculate score

Table 6.1 Non-invasive scores for predicting non-alcoholic fatty liver disease

Score	Markers evaluated	Coorte (no of patients)	Sensitivity (%)	Specificity (%)	Cut-off	AUC	Reference
FLI	BMI, waist circumference, GGT	396	87	86	>60	0.84	Bedogni et al., 2006 [15]
Kotronen score	Metabolic Syndrome, type 2 diabetes, fasting serum insulin levels, AST, AST/ALT ratio	470	86	71	−0.64	0.87	Kotronen et al., 2009 [17]
LAP	Waist circumference, triglycerides	588	77[a]	75[a]	30.5[a]	0.80	Bedogni et al., 2010 [18]
			82[b]	79[b]	23.0		
Korean study score	AST/ALT ratio, GGT, triglycerides, BMI	900	71.7	75.9	>3	0.79	Young et al., 2011 [19]
HSI	AST/ALT ratio, BMI, gender, type 2 diabetes	10,000	93	92	>36	0.81	Lee et al., 2010 [20]
Steatotest	a-MG, haptoglobin, apolipoprotein A1, total bilirubin, GGT or ALT, fasting glucose, triglycerides, cholesterol, age, gender, BMI	800	90	70	0.3	0.89	Poynard et al., 2005 [21]

Abbreviations: *a-MG* alpha-2 macroglobulin, *ALT* alanine transaminase, *AST* aspartate aminotransferase, *AUC* area under the curve, *BMI* body mass index, *FLI* fatty liver index, *GGT* gamma-glutamyl transpeptidase, *HIS* hepatic steatosis index, *LAP* lipid accumulation product

[a]Men

[b]Women

including body mass index (BMI), waist circumference, gamma-glutamyl transferase (GGT), and serum triglycerides levels. It was developed in a population of 396 patients with US diagnosis of steatosis, showing an area under curve (AUC) of 0.84 for the detection of fatty liver. Ranging from 0 to 100, a FLI <30 allows to rule out, while a FLI >60 detects accurately fatty liver. Its good performance was further confirmed in other populations [16, 23], so FLI could be used to identify patients for US and for intensification of lifestyle measures. The Kotronen score is based on a combination of clinical (metabolic syndrome, type 2 diabetes) and serum markers (fasting serum insulin levels, aspartate transaminase [AST], AST/ALT ratio) and it was validated using spectroscopy MR as reference standard method, differently from other scores that utilised US. AUC was 0.87 in the estimation and 0.86 in the validation group [17]. LAP results from a combination of waist circumference and triglycerides and it showed an AUC of 0.80 for the prediction of steatosis [18] and an AUC of 0.72 in a subsequent validation study [16]. A Korean study [19] conducted in a cohort of about 900 patients evaluated a score including AST/ALT ratio, GGT, triglycerides and BMI, with an AUC of 0.797 for prediction of US steatosis. Similarly, HSI was elaborated and internally validated in a cohort of about 10,000 Korean subjects with US-diagnosed NAFLD, showing an AUC of 0.812 and a direct relationship with US severity of steatosis [20]. It includes AST/ALT ratio, BMI, gender and type 2 diabetes. Finally, the Steatotest [21] was studied and validated in a population of about 800 patients with histologic evidence of chronic liver disease correlated with different etiologies, showing an AUC of 0.89 for detection of steatosis >5% and an AUC ranging from 0.72 to 0.86 in the validation cohorts. More recently, an individual patient data meta-analysis showed a weighted AUC of 0.80 for the detection of steatosis >30% [22]. This score is composed by haptoglobin, a2-macroglobulin, apolipoprotein A1, total bilirubin and GGT, BMI, cholesterol, triglyceride, and glucose, corrected for age and gender.

Although the data presented above had showed a good diagnostic performance (especially for the FLI, that was validated in population studies and for the Kotronen score, that was elaborated using spectroscopy RM as diagnostic reference), it should be underlined that these algorithms/scores are useful mainly for epidemiological studies, but they have a relative usefulness in the single patient. Furthermore, an external validation is needed to demonstrate their diagnostic performance in other clinical scenarios.

Detection of NASH

The occurrence of NASH, and eventually the development of fibrosis, have a clinically relevant impact on the prognosis of NAFLD patients in regards of both hepatic and extrahepatic morbidity and mortality, as showed in natural history studies. NASH compared with simple steatosis is associated with a faster progression of fibrosis [24] and, not surprisingly, with a higher risk of cirrhosis and death. The severity of fibrosis, promoted by the presence of steatohepatitis, was showed to be

the strongest predictor of liver-related complications, cardiovascular diseases and extrahepatic neoplasms [25]. It is clear that the correct identification of NAFLD patients between those with simple steatosis and those with NASH and/or fibrosis is relevant for prognostic stratification and, subsequently, for therapeutic decisions.

To date, the gold standard for the discrimination between simple steatosis and NASH (steatosis, hepatocyte ballooning and lobular inflammation) and for the staging of fibrosis remains the liver biopsy. However, to perform liver biopsy in all the NAFLD patients would be unfeasible and unethical, considering its invasiveness, the potential occurrence of life-threatening complications, and the high prevalence of NAFLD in general population. Furthermore, liver biopsy has some limitations in the diagnostic accuracy mainly related to sampling errors and to intra- and interobserver reproducibility, although histological scores/algorithms have been developed to decrease disagreement among pathologists [26].

Several studies have been conducted to identify non-invasive methods for the detection of NASH and fibrosis, although this still remains an unmet medical need because there are no available clinical and/or biochemical tests to distinguish NASH from simple steatosis in clinical practice. Promising results have been showed for cytokeratin-18 (CK-18) serum fragments, a marker of apoptosis, that has been found to be a relevant pathogenic mechanism involved in NASH [27]. CK-18 fragments have been shown to be significantly higher in patients with NASH compared to those with simple steatosis, with an AUC ranging from 0.78 [28] to 0.93 [27, 29, 30]. A meta-analysis of nine studies including 852 patients confirmed these promising data, showing a pooled AUC of 0.82 for the prediction of NASH [31]. The diagnostic performance of CK-18 was also evaluated in combination with other biomarkers, namely Adipocyte Fatty Acid Binding Protein (AFABP) and Fibroblast Growth Factor 21 (FGF21), in patients with histologically proven NAFLD, demonstrating an improvement of the diagnostic accuracy using a two-step approach with CK-18 and FGF21 [32]. Similarly, the combination of CK-18 with serum adiponectin and resistin showed an accurate prediction of NASH (AUC of 0.85 in the overall cohort, 0.90 in the training group and 0.73 in the validation group) [33], as well as the combination with other marker of apoptosis, like soluble Fas levels, that improved diagnostic accuracy in comparison with that of each biomarker taken individually (AUC of 0.93 in a training set of patients with biopsy-proven NAFLD and 0.79 in a validations set of patients who underwent biopsy during bariatric surgery) [34]. Furthermore, CK-18 was studied also in combination with ALT and the presence of the metabolic syndrome (the so-called Nice model) in a cohort of morbidly obese patients underwent to bariatric surgery, showing an AUC of 0.88 and 0.83 in training and validation group, respectively [35]. More interestingly, some studies compared the performance of traditional CK-18 levels fragments (cleaved) using M30 ELISA with total CK-18 levels (cleaved and uncleaved) using M65ED ELISA, that is not only a marker of apoptosis but also of necrosis [36]: diagnostic performance was better for total CK-18 than for fragments, with an AUC of 0.93 and 0.77 respectively, in the detection of NASH, independently of ALT levels. By contrast, a large multicentre cohort study including more than 400 patients with biopsy proven NAFLD showed a low diagnostic accuracy for NASH, with an AUC of 0.65, with

a significant overlap of CK-18 levels between patients with NASH and those with simple steatosis [37]. Similarly, a recent meta-analysis showed a pooled sensitivity of 66% and specificity of 82% in diagnosing NASH, suggesting that CK-18 fragments could have a modest usefulness in clinical practice [38]. These contrasting data reduced the initial enthusiasm placed towards the use of CK-18 for the detection of NASH, that can not to be recommended in clinical practice, considering the lack of a well standardized test and the uncertainty in the definition of diagnostic thresholds.

Serum panels based on the combination of clinical and biochemical features with pathogenic serum markers of NASH are another useful tool to identify NASH noninvasively. However, their use in clinical practice is limited by the absence of validation data and so they are not recommended (Table 6.2). NASH test combining 13 parameters (age, sex, height, weight, triglycerides, total cholesterol, α2-macroglobulin, apolipoprotein A1, haptoglobin, GGT, ALT, AST, and total bilirubin) showed an AUC of 0.79 in the original group, both in the training and in validation groups [39]. NASH test was further validated in a population of patients undergoing to bariatric surgery, with similar results (AUC 0.77) [40]. The diagnostic accuracy of NASH test was further evaluated by an individual patient data meta-analysis that pooled data of other independent studies, demonstrating an AUC of 0.84 [22]. Index of NASH (ION) score was obtained combining waist-to-hip ratio, triglycerides, ALT and Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) and it demonstrated an AUC of 0.88 in a cohort of 152 patients with biopsy proven NAFLD [41], although data from a multicentre Italian study on 292 patients with NAFLD did not confirm the good diagnostic performance of ION for detection of NASH (AUC 0.68) [42]. Some studies evaluated the diagnostic accuracy of the combination of serum hyaluronic acid (HA) with other clinical and biochemical biomarkers, like age, gender, AST, AST/ALT ratio and BMI, showing an AUC of 0.76 [43]. Similarly, an Italian study elaborated a model that included clinical variables (i.e., age) and serum markers of inflammation and fibrosis (i.e. HA and tissue inhibitor of metalloproteinases 1 [TIMP-1]) showing an AUC of 0.93, although it was obtained in a small cohort of 46 NAFLD patients [44]. NAFIC score was obtained in a cohort of 177 biopsy-proven NAFLD patients and validated on 442 patients from Japan. The score included serum ferritin, fasting insulin and type IV collagen 7S, showing an AUC for the detection of NASH of 0.85 in the training group and 0.78 in the validation group [45]. Other studies evaluated the combination between serum adiponectin levels, that have been showed to be reduced in patients with NASH in comparison with those with simple steatosis, with other biochemical markers, i.e. HOMA-IR and type IV collagen 7S [46], and subsequently with HOMA-IR alone, demonstrating an acceptable diagnostic performance (AUC 0.79) [47]. A French study combined adiponectin/leptin ratio with HOMA-IR, demonstrating an AUC of 0.82 in distinguishing NASH from simple steatosis [48]. In addition to adipokines, other studies addressed the use of cytokines for the noninvasive diagnosis of NASH, like interleukin-6 (IL-6), that have been showed to predict NASH with an AUC of 0.81 [49], and serum interleukin 1 receptor antagonist (IL-1 RA) in combination with AST and fasting insulin [50], that demonstrated

Table 6.2 Non-invasive scores for predicting non-alcoholic steatohepatitis in patients with non-alcoholic fatty liver disease

Score	Markers evaluated	Coorte (no of patients)	Sensitivity (%)	Specificity (%)	Cut-off	AUC	Reference
NASH test	a-MG, haptoglobin, apolipoprotein A1, total bilirubin, GGT, ALT, AST, triglycerides, cholesterol, age, gender, height, weight	257	33	94	0.75	0.79	Poynard et al., 2006 [39]
ION	Waist-to-hip circumference ratio, triglycerides, ALT, HOMA-IR	152	60	92	≥60	0.88	Otgonsuren et al., 2014 [41]
NAFIC score	Serum ferritin, fasting insulin and type IV collagen 7S	177	60	87	2	0.85	Sumida et al., 2011 [45]
HAIRE score	Hypertension, ALT, HOMA-IR	105	80	89	≥2	0.9	Dixon et al., 2011 [55]
NASH score	PNPLA3 genotype, AST, fast insulin	296	75	75	−1.054	0.77	Hyysalo et al., 2014 [58]
NASH ClinLipMet score	PNPLA3 genotype, glutamate, isoleucine, glycine, lysophosphatidylcholine 16:0, phosphoethanolamine 40:6, AST, fasting insulin	318	85.5	72	0.134	0.86	Zhou et al., 2016 [59]

Abbreviations: *a-MG* alpha-2 macroglobulin, *ALT* alanine transaminase, *AST* aspartate aminotransferase, *AUC* area under the curve, *BMI* body mass index, *GGT* gamma-glutamyl transpeptidase, *HOMA-IR* homeostasis model assessment for insulin resistance, *ION* index of NASH

to be able to accurately predict inflammation scores. On the other side, some authors have emphasized the role of oxidative stress and its biomarkers, namely the thioredoxin, that was showed to be higher in patients with NASH in comparison with healthy controls and patients with simple steatosis, with an AUC of 0.78, although these results were obtained in a small cohort of 57 patients [51]. The impact of inflammatory biomarkers, particularly the neutrophil/lymphocyte (N/L) ratio, was assessed in a cohort of 101 consecutive patients who underwent to liver biopsy for clinical suspicion of NAFLD showing that for each one-unit increase in N/L ratio, the risk of having NASH increased by 70% [52]. Some studies evaluated the role of other metabolic alterations in the prediction of NASH, like hyperuricemia and ferritin levels. In particular, an Italian study found that hyperuricemia was an independent predictor of NAFLD Activity Score (NAS) > 5 in a cohort of 166 patients with histologically proven NAFLD, together with female gender and higher HOMA-IR [53]. On the other side, another study conducted in a large cohort of 600 patients with histologically proven NAFLD showed that high serum ferritin levels, that are a well-known marker of systemic inflammation, were significantly associated with NASH, independently of the presence of iron deposition in the liver [54]. Other studies were performed in NAFLD patients with severe obesity. A combination of hypertension, ALT and HOMA-IR (the HAIR score) was associated with an AUC of 0.90 in a cohort of 105 severely obese patients underwent to gastric bypass surgery [55]. In a similar clinical setting of severely obese NAFLD patients undergoing bariatric surgery, a simple model including AST and diabetes predicted NASH with an AUC of 0.82 [56]. Finally, to reduce unnecessary diagnostic liver biopsy in morbidly obese patients, an US study elaborated a score including arterial hypertension, AST, ALT, sleep apnoea, type 2 diabetes and non-Black race, identifying thresholds for low, intermediate, high and very high probability of NASH [57].

The combination of clinical and biochemical markers with genetic variables is an interesting and promising way to improve diagnostic accuracy in the detection of NASH. The NASH score has been studied in a cohort of 296 morbid obese Finnish patients undergoing to liver biopsy during bariatric surgery and it includes PNPLA3 genotype, AST and fasting insulin [58]. It was validated in a cohort of 380 Italian patients with biopsy proven NAFLD, demonstrating an AUC of 0.77 and 0.76 in training and validation groups, respectively. The same authors further improved the overall diagnostic accuracy of this score with the addition of other five variables identified by mass spectroscopy in the serum (glutamate, isoleucine, glycine, lysophosphatidylcholine 16:0, phosphoethanolamine 40:6), obtaining the NASH ClinLipMet score, that showed a better diagnostic performance (AUC 0.86) in comparison to the original NASH score [59].

Finally, in front of the great number of score and panels developed in the studies above presented, unfortunately today clinicians do not have accurate and user-friendly tools for the distinction between NASH and simple steatosis. The lack of availability of some of the variables included in many scores is one of the reasons that limit their use in clinical practice, along with not enough high accuracy. The combination with genetic variables, with imaging techniques or with “omics” approaches may allow in the future a better diagnostic performance for identification of patients with NASH.

Detection and Assessment of Fibrosis

While the non-invasive identification of NASH remains an unmet medical need, a greater number of evidences are available for the non-invasive prediction of fibrosis. As already stated, in patients with NAFLD, fibrosis stage is the most clinically relevant predictor of overall and disease-specific, as showed by two long-term natural history studies [7, 8]. For this reason, several non-invasive panels including demographic, serum and fibrosis markers, have been studied to assess the severity of liver fibrosis in NAFLD, with an AUC ranging from 0.56 to 0.93 for the detection of severe liver fibrosis, but with a poor accuracy for the detection of the lower stages of fibrosis.

Demographic and Serum Markers

Demographic and serum markers include easily available variables, easy to apply in clinical practice, and they reflect the main risk factors associated with the presence of liver fibrosis, although they do not directly reflect the mechanisms associated with fibrogenesis and fibrinolysis. The most studied non-invasive scores for the assessment of fibrosis severity are NAFLD Fibrosis score (NFS), Fibrosis-4 (FIB-4), BARD score, AST to PLT ratio (APRI), AST to ALT ratio (AAR), FibroMeter, eLIFT and HEPAMET score (Table 6.3). Among these, the most validated and best performing tools, especially in ruling-out advanced fibrosis, are represented by NFS and FIB-4.

NFS is calculated using a pre-defined formula that includes six easily available variables (age, BMI, AST/ALT ratio, platelet count, hyperglycemia and albumin). It was designed in a large multinational multicenter cohort of 733 patients with biopsy proven NAFLD, showing an AUC of 0.84 for the detection of severe fibrosis [60]. NFS uses two diagnostic thresholds, one for rule-in (0.676, with 67% sensitivity and 97% specificity) and one for rule-out advanced fibrosis (−1.455, with 90% sensitivity and 60% specificity) [31]. Similar results were observed in other European studies: an UK study demonstrated an AUC of 0.81 for advanced fibrosis in 145 consecutive patients with biopsy proven NAFLD [61] and similarly, a French study showed an AUC of 0.88 for significant fibrosis, 0.93 for severe fibrosis and 0.90 for cirrhosis in 235 NAFLD patients with METAVIR histology staging [62]. NFS was also evaluated in Asiatic NAFLD patients and in patients with severe obesity undergoing bariatric surgery, showing a similar good diagnostic performance for the detection of significant and/or advanced fibrosis [63–65]. Finally, a recent meta-analysis confirmed the good diagnostic accuracy of NFS in detecting significant fibrosis (AUC 0.72 in 11 studies), severe fibrosis (AUC 0.78 in 38 studies) and cirrhosis (AUC 0.83 in 8 studies) [66]. Interestingly, a retrospective international multicenter cohort study of more than 320 patients with biopsy proven NAFLD showed a promising role of NFS in the prediction of long-term outcomes of these patients: stratifying the study population in three risk groups according to baseline NFS,

Table 6.3 Non-invasive scores for predicting liver fibrosis in patients with non-alcoholic fatty liver disease

Score	Markers evaluated	Diagnostic endpoint/s	Coorte (no of patients)	Cut-off	Sensitivity (%)	Specificity (%)	Overall population AUC	Reference
NAFLD fibrosis score	Age, BMI, AST/ALT ratio, platelet count, hyperglycemia, albumin	Severe fibrosis	733	≤ −1.455	82	77	0.88	Angulo et al., 2007 [60]
				≥ 0.676	51	98		
FIB-4	Age, AST/platelet count, ALT	Severe fibrosis	145	1.3	85	65	0.86	McPherson et al., 2010 [61]
				3.25	26	98		
BARD	BMI ≥ 28 kg/m^2, AST/ALT ratio ≥0.8, presence of T2DM	Severe fibrosis	827	<2	62	66	0.81	Harrison et al., 2008 [70]
APRI	AST/PTL	Significant fibrosis	235	–	–	–	0.86	Cales et al., 2009 [62]
		Severe fibrosis					0.86	
		Cirrhosis					0.84	
AAR	AST/ALT	Severe fibrosis	145	0.8	74	78	0.83	McPhersonh et al., 2010 [61]
				1	52	90		
FibroMeter	Age, body weight, fasting glucose, AST, ALT, ferritin, platelet count	Significant fibrosis	235	–	–	–	0.94	Cales et al., 2009 [62]
		Severe fibrosis					0.93	
		Cirrhosis					0.9	
eLIFT	Age, gender, GGT, AST, platelet count, prothrombin time	advanced fibrosis	3754	≥8	78	–	0.78	Bousier et al., 2017 [75]
		Cirrhosis			94	–	0.85	

HEPAMET score	Female gender, age, HOMA index, diabetes, AST, albumin, platelet count	Fibrosis (F2)	1337	12	58	84	0.78	Ampuero et al., 2018 [76]
				24	41	94		
		Fibrosis (F3)		12	80	83	0.86	
				24	74	88		
		Cirrhosis (F4)		12	96	77	0.92	
FibroTest	Haptoglobin, a2-MG, apolipoprotein A1, total bilirubin, GGT	Advanced fibrosis	267	0.3	92	71	0.88	Ratziu et al., 2006 [116]
				0.7	25	97		
Hepa score	Age, gender, a2-MG, bilirubin, GGT, hyaluronic acid	Significant fibrosis	242	0.37	75	84	0.72	Adams et al., 2011 [87]
		Advanced fibrosis					0.81	
		Cirrhosis					0.9	
ELF	Age, P3NP/PIIINP, hyaluronic acid, TIMP-1	Any fibrosis	192	−0.1068	90	75	0.82	Guha et al., 2008 [81]
		Significant fibrosis					0.9	
		Severe fibrosis		0.3576	80	90	0.93	
ADAPT	Age, diabetes, PRO-C3, platelet count	Advanced fibrosis	281	–	–	–	0.87	Daniels et al., 2019 [86]
MAK32	AST, HOMA index, CK-18	Advanced fibrosis	846	≥0.550	90	95	0.84	Bousier et al., 2018 [93]

Abbreviations: *ALT* alanine transaminase, *APRI* AST-to-PLT ratio index, *ARR* AST to ALT ratio, *AST* aspartate aminotransferase, *AUC* area under the curve, *BMI* body mass index, *CK-18* cytokeratin-18, *ELF* European liver fibrosis, *FIB-4* fibrosis-4, *GGT* gamma-glutamyl transpeptidase, *HOMA-IR* homeostasis model assessment for insulin resistance, *P3NP/PIIINP* N-terminal peptide of procollagen III, *TIMP-1* tissue inhibitor of matrix metalloproteinase 1, *T2DM* type 2 diabetes mellitus

authors showed that NFS was significantly associated with the risk of occurrence of liver-related events and with the risk of death or liver transplantation. These results are relevant because they give to NFS a role not only for the diagnosis and the assessment of the fibrosis, but also for the prognosis [67]. Considering the available evidences, the American Association for the Study of Liver Disease (AASLD) 2012 Guidelines stated that NFS is a clinically useful tool for identification of NAFLD patients at higher likelihood to have bridging fibrosis and/or cirrhosis [14].

The FIB-4 arises from the combination of age, platelet count, AST and ALT and it has a similar diagnostic accuracy compared to NFS [61], with an AUC of 0.86 for advanced fibrosis. These data were also confirmed in a cohort of 576 Japanese histologically proven NAFLD patients, with an AUC of 0.87 [68]. A recent meta-analysis [66] reported an AUC of 0.75 for significant fibrosis (12 studies), 0.80 for severe fibrosis (34 studies) and 0.85 for cirrhosis (8 studies). Similarly to NFS, there are two diagnostic thresholds for rule-in (2.67) and rule-out (1.3) advanced fibrosis. Interestingly, it was recently observed in a large multicenter cohort of more than 600 patients with biopsy proven NAFLD that these thresholds for diagnosis of advanced fibrosis do not perform so well in patients older than 65 years, suggesting new cut-offs to improve specificity [69].

BARD and APRI are other simple to use non-invasive scores elaborated for the detection and the assessment of fibrosis severity in NAFLD patients. In particular, BARD combines BMI, AST/ALT ratio and type 2 diabetes with an acceptable diagnostic accuracy reported both in European [70, 71] and Asian populations [63]. APRI was initially evaluated in patients with chronic hepatitis C and subsequently in NAFLD with contrasting results: a French study [62] showed a good diagnostic performance with an AUC of 0.87 for significant fibrosis, 0.86 for severe fibrosis and 0.84 for cirrhosis, but these results were not confirmed in a South American study [72], including only 30 patients with NAFLD, that showed an AUC of 0.56 for significant fibrosis, 0.57 for advanced fibrosis and 0.79 for cirrhosis. Anyway, diagnostic accuracy of BARD and APRI is significantly lower in comparison with that of NFS and FIB-4, as demonstrated by a meta-analysis reporting an AUC of 0.70 for significant fibrosis and 0.75 for severe fibrosis and cirrhosis, regarding APRI, and an AUC of 0.64 for significant fibrosis, 0.73 for severe fibrosis and 0.70 for cirrhosis, regarding BARD [66]. The superiority of NFS and FIB-4 on the other non-invasive models for the detection of advanced fibrosis was also reported in a recent retrospective study of 1904 biopsy proven NAFLD patients, with an AUC of 0.78 and 0.80, respectively [73].

FibroMeter included age, body weight, fasting glucose, AST, ALT, ferritin and platelet count and it was assessed in a French cohort of 235 patients with NAFLD [62]. Diagnostic accuracy observed was good, with AUC of 0.94 for significant fibrosis, 0.93 for severe fibrosis and 0.90 for cirrhosis. Similar results were observed in a cohort of 145 patients with histologically proven NAFLD: AUCs were 0.80 for any stage of fibrosis and 0.86 for severe fibrosis [74].

Overall, NFS, FIB-4 and the other non-invasive scores are limited by the relevant rate of false positive results, and the high rate—up to around 50%—of patients falling in the grey zone, that is an area of diagnostic uncertainty of the test. Recent

studies showed promising results in the improvement of diagnostic accuracy of non-invasive scores. The easy liver fibrosis test (eLIFT) score [75] showed a similar sensitivity for the detection of advanced fibrosis in comparison with FIB-4, but with a lower false positive rate in the validation group, suggesting a potential role as screening test. This score is composed by age, gender, GGT, AST, platelet count and prothrombin time and it was derivated and validated in a cohort of 3754 patients with biopsy proven chronic liver disease. Finally, data on diagnostic accuracy of the Hepamet score were presented at International Liver Congress 2018 [76]. This score was assessed in an international study of 1337 biopsy proven NAFLD patients from Spain, Italy and USA. It includes female gender, age, HOMA index, diabetes, AST, albumin and platelet count. AUROC for severe fibrosis and cirrhosis were respectively 0.86 and 0.92. Interestingly, when compared with FIB4 and NFS, Hepamet score was associated with a low rate of unclassified patients falling in the grey zone and with a higher rate of correct classification of the fibrosis stage.

In consideration of the great number of non-invasive scores evaluated during last decade, some studies tried to compare their performance. An already mentioned French study [62] conducted in 235 NAFLD patients compared the performances of FibroMeter, NFS and APRI for the detection of significant fibrosis, showing a highest accuracy by using FibroMeter (AUC 0.94), followed by NFS (AUC 0.88) and APRI (AUC 0.86). According to the authors, the use of FibroMeter could allow to avoid liver biopsy in about 97% of patients. Similarly, a USA study [77] conducted in a large cohort of 541 patients with histologically proven NAFLD showed a higher accuracy for the diagnosis of severe fibrosis by using FIB-4 (AUC 0.80) in comparison with NFS (AUC 0.76), APRI (AUC 0.73) and BARD score (AUC 0.70). These data were substantially confirmed in an UK cohort study [61] of 145 patients with biopsy proven NAFLD, showing an AUC of 0.86 for the diagnosis of advanced fibrosis, that was higher in comparison with NFS (AUC 0.81), BARD (AUC 0.77) and APRI (AUC 0.67). Similarly, a large cohort study enrolling Japanese NAFLD patients [68] showed that FIB-4 had an AUC of 0.87 for the diagnosis of advanced fibrosis, that resulted better if compared with NFS (AUC 0.86) and APRI (AUC 0.82).

Fibrosis Biomarkers

Differently from demographic and serum biomarkers, fibrosis biomarkers directly reflect the mechanisms of fibrogenesis and fibrinolysis, but they are more complex and expensive, resulting more difficulty applicable to the large NAFLD population. Among these, the best validated is the FibroTest [78], a panel that includes five biochemical markers (haptoglobin, a2-macroglobulin, apolipoprotein A1, total bilirubin and GGT) adjusted for age and gender. It was developed to assess fibrosis in different chronic liver disease, including NAFLD. Two meta-analyses [22, 78] were performed: the first [78] showed a mean standardized AUC of 0.84 for advanced fibrosis; the second, using individual data from 494 NAFLD patients

with severe obesity, showed an AUC of 0.85 for severe fibrosis. Similar results were also reported in two more recent studies: a French study [79] conducted on 452 NAFLD patients (AUC of 0.75 for F2–F4, 0.77 for F3–F4 and 0.80 for F4) and an international multicenter study of 600 patients [80], showing non binary AUROC of 0.87 for fibrosis stages. This last study also compared FibroTest with other non-invasive biomarkers, demonstrating a higher diagnostic performance in comparison with BARD (non binary AUROC 0.83) and FIB-4 (non binary AUROC 0.84), but no significant differences were observed when FibroTest was compared with NAFLD fibrosis score (non binary AUROC 0.86. p = 0.26). Other than FibroTest, other non-invasive scores including fibrosis biomarkers were developed but they received less validation compared to FibroTest. Hepascore was developed in a French cohort study [79] and it included age, gender, alpha2-macroglobulin, bilirubin, GGT and hyaluronic acid. AUCs observed were 0.75 for significant fibrosis, 0.77 for advanced fibrosis and 0.80 for cirrhosis. The European Liver Fibrosis (ELF) test was used in UK and it is composed by the combination of age, N-terminal peptide of procollagen III (P3NP/PIIINP), hyaluronic acid and tissue inhibitor of matrix metalloproteinase 1 (TIMP-1). In a cohort of 192 biopsy proven NAFLD patients, AUCs for severe fibrosis and moderate fibrosis were 0.90 and 0.82 respectively, using a simplified panel obtained excluding age [81]. Indeed, a component of ELF, particularly hyaluronic acid, alone or combined with type VI collagen 7S domain has been showed to have a good diagnostic accuracy for severe fibrosis, with AUCs ranging from 0.75 to 0.97 [82–85]. Finally, a recently proposed algorithm [86] based on the combination of age, diabetes, PRO-C3 (a marker of type III collagen formation) and platelet count, named ADAPT, showed an AUROC for the diagnosis of advanced fibrosis of 0.86 and 0.87 in the derivation (150 patients) and in the validation group (281 patients), respectively. Interestingly, ADAPT showed a higher diagnostic accuracy when compared with NFS, FIB-4 and APRI, although a further validation is needed.

In consideration of the several non-invasive biomarkers of liver fibrosis, the accuracy of simple (BARD, APRI) versus complex fibrosis models (FIB-4, FibroTest, Hepascore) was compared in a multicentre cohort of 242 patients with NAFLD. Regarding significant fibrosis, accuracy was modest for all the panels (AUCs ranging from 0.70 to 0.74), while complex models had a better diagnostic performance for advanced fibrosis and cirrhosis, in comparison with simple models [87]. Overall, diagnostic accuracy of serum fibrosis panels for staging fibrosis is similar, if not higher, to that showed for the non-invasive scores, but their cost, being patented markers, reduces the potential application in clinical practice.

Combination Strategies

Considering the diagnostic limitations above mentioned regarding the non-invasive assessment of liver fibrosis, last European NAFLD guidelines stated that the combination of non-invasive biomarkers and scores with transient elastography

could improve diagnostic accuracy, in order to reduce the number of diagnostic liver biopsies [3]. For these reason, several studies evaluated the association of non-invasive scores with liver stiffness measurement (LSM) by FibroScan or the association of two non-invasive scores. An UK study [81] assessed the combination of ELF with NFS, showing an improvement of diagnostic accuracy in comparison with ELF alone, with AUCs of 0.98 for severe fibrosis, 0.93 for moderate fibrosis and 0.84 for no fibrosis. This combination could allow to avoid unnecessary liver biopsies in 88% of patients. Interestingly, FibroMeter in combination with LSM was investigated in a French study of 225 NAFLD patients, showing a better diagnostic performance in comparison to that of FibroMeter alone [88]. The combination of LSM with NFS or FIB-4 was assessed in 321 Italian patients, showing an improvement in the diagnostic accuracy for severe fibrosis, although about half of patients remained unclassified [89]. To limit the rate of patients falling in the grey zone, some authors proposed the use of serial assessment of LSM and non-invasive scores. Particularly, an International multicenter study conducted in Sicily (Italy), France, Wales and Hong Kong assessed the serial combination of NFS or FIB-4 with LSM, showing that this approach consisting in the use of a second test (LSM) in patients falling in the grey zone at the first test increases the diagnostic performance with an accuracy of about 76–78%, a rate of wrong classification of about 15% and an uncertainty area <8% [90]. This approach resulted more useful than the paired combination of LSM with NFS or FIB-4, that generated a wide uncertainty area, including more than half of patients, although the rate of diagnostic errors was lower than 3%. Similarly, a French study [75] conducted in patients with chronic liver diseases from different etiologies evaluated an algorithm based on eLIFT as first-line and the combination of FibroMeter with vibration controlled transient elastography (VCTE) as second-line in patients with high eLIFT score, showing a sensitivity of 76% for advanced fibrosis and 92% for cirrhosis. A recent study [91] on a cohort of 968 biopsy proven NAFLD patients confirmed the superiority of a serial combination strategy consisting in NFS or FIB-4 with LSM to the use of a single test and it assessed the impact of obesity and AST levels on the diagnostic accuracy of the combination of these tools. Particularly, authors found that the serial combination strategy improved diagnostic accuracy in all subgroups, but it had a higher accuracy in non obese patients and a lower accuracy in obese ones. By contrast, ALT levels did not interfere with the diagnostic performance of non-invasive scores, although LSM resulted superior to both NFS and FIB-4 in obese and/or low ALT patients. More recently, preliminary data presented during the last International Liver Congress showed a potential role of a stepwise algorithm combining Hepamet score and LSM, that showed a diagnostic accuracy higher than 98% for the diagnosis of advanced fibrosis, although it was applicable only in 45% of the overall study population [92]. In conclusion, to date, the strategy to serially combine non-invasive scores (NFS or FIB-4) with LSM in patients falling in the uncertainty area is suggested by European guidelines for its acceptable accuracy and reliability, especially to rule out advanced fibrosis.

Detection of NASH with F2–F4

Randomized controlled trials assessing the efficacy of pharmacological treatments in NAFLD often include patients with histological diagnosis of NASH and significant fibrosis. However, above mentioned non-invasive scores were mainly developed to identify patients with severe fibrosis. To identify patients with NAFLD activity score (NAS) ≥4 and fibrosis stage ≥F2 (so-called fibrotic NASH), MACK-32 was developed in a large cohort of 846 French and Belgian NAFLD patients [93]. It is composed by a combination of AST, HOMA index and CK-18 serum fragments and it was compared with other non-invasive scores. Interestingly, diagnostic accuracy for fibrotic NASH of NFS, FIB-4 and BARD was poor, while MACK-32 showed an AUC of 0.847 in the validation set. However, the low availability of HOMA index and CK-18 in clinical practice represents a limit for this tool. In this line, data on the external validation of the combination of AST with FibroScan LSM and CAP were presented during the last International Liver Congress showing a good diagnostic accuracy for identification of patients with NAS ≥4 and fibrosis ≥F2, with a pooled AUC of 0.82, suggesting a potential role of this simple score to screen patients for drugs trials [25].

Assessment of the Prognosis

Ideally, non-invasive biomarkers should inform clinical decision making predicting the prognosis, that in NAFLD patients is mainly related to the fibrosis progression, the main driver toward the occurrence of liver-related and unrelated events [7, 8, 94]. Although no specific biomarkers have been evaluated with this aims, some studies assessed the prognostic role of non-invasive biomarkers developed for the assessment of the severity of the liver disease. In NAFLD patients, the progression of liver fibrosis is not linear and it could be slower or faster in some patients, although no data are available about non-invasive biomarkers able to distinguish slow from fast progressors. A recent longitudinal study assessed the ability of non-invasive scores to detect changes in fibrosis stage over time in 292 histologically proven NAFLD patients who repeated liver biopsy after a median time interval of 2.6 years from baseline. Changes in longitudinal assessment of APRI, FIB-4 and NFS resulted significantly associated with fibrosis progression. In particular, FIB-4 and NFS had the greatest accuracy in the prediction of the progression to advanced fibrosis, with high negative predictive values (NPV), but suboptimal positive predictive values (PPV) [73]. Indeed, some metabolic comorbidities were identified as risk factors for fibrosis progression. A small study on 52 Asiatic patients undergoing to a second liver biopsy after 3 years showed that modifications of BMI and waist circumference were independently associated with a progressive or non-progressive course of the disease and fibrosis [95]. An Italian study [96] identified incident arterial hypertension and HOMA-index as risk factors for fibrosis progression, while diabetes has been showed to be a significant predictor of liver fibrosis [97, 98], also in patients with simple steatosis [99].

Some studies tested the predictive ability for liver and not-liver related outcomes of the scores above mentioned and developed for fibrosis assessment. A large cohort of 4083 patients with an US based diagnosis of NAFLD conducted in USA showed that those with a NFS score suggesting advanced fibrosis had higher overall, liver-related and cardiovascular mortality [100]. Similar results were reported in a retrospective cohort study of 320 patients with histologically proven NAFLD, in whom NFS is able to predict liver events and overall mortality, with higher accuracy in comparison with other non-invasive scores, like APRI, BARD and FIB-4 [67] and in a French study [79] that compared APRI, FIB-4, Hepascore, FibroMeter and FibroScan. Interestingly, FibroMeter and FibroScan were able to categorise NAFLD patients into subgroups with significantly different prognosis, suggesting a potential role of FibroMeter in the prediction of the mortality in NAFLD patients.

According to data above presented, non-invasive biomarkers assessing the severity of liver fibrosis could help to stratify NAFLD patients according their prognosis, although further studies are needed to develop specific biomarkers of prognosis and to assess the impact of dynamic changes in fibrosis stages on the long-term outcomes.

Assessment of the Response to Treatments

Pharmacological treatment of NASH represents an unmet medical need considering that to date no medication has been approved by Food and Drug Administration and by European Medicines Agency for treatment of NAFLD, In spite of the increasing epidemiological burden worldwide. The aims of the treatment should be the reduction of liver-related mortality and progression towards cirrhosis and its complications, so the identification of adequate non-invasive surrogate markers is important to assess the effectiveness of new drugs evaluated for the treatment of NASH.

Although several pharmacological treatments have been assessed, to date weight loss remains the only treatment for which solid evidences have been provided. Interestingly, weight loss, obtained both with lifestyle changes or with bariatric surgery, induces disappearance of NASH and improvement of fibrosis in a "dose-dependent" manner, i.e. the greater the weight loss (no less than 5–7%), the greater the improvement of NASH histological lesions [101, 102]. Anyway, several clinical trials are evaluating molecules targeting different pathogenic pathways of NASH, with promising results [103]. For this reason, when effective pharmacological treatment for NASH will be approved by regulatory agencies, we will need to have non-invasive biomarkers to correctly identify responders from non-responder patients, in such a way as to best personalize the treatment, to increase utility and benefit and to reduce risk. Unfortunately, only few data are available about the use of non-invasive biomarkers in the assessment of the response to treatments.

It was suggested that the rs738409 C → G single nucleotide polymorphism of PNPLA3, that is associated with a higher risk of NAFLD, could influence the ability of reduce fatty liver after weight loss. In particular, a small study conducted in 18

patients showed that those carrying the PNPLA 3 G allele had a significantly higher reduction in fatty liver (evaluated by spectroscopy MR) after weight loss induced by hypochaloric and low-carbohydrate diet [104]. These results were confirmed by a post-hoc analysis of a randomized controlled trial on lifestyle modifications that showed a higher sensitivity to the beneficial effects of lifestyle modifications in patients with PNPLA3 G allele [105], particularly in the reduction of intrahepatic triglyceride content assessed with spectroscopy MR. However, these studies were limited by the lack of data about the improvement of liver damage. More recently, a retrospective analysis of the data collected in the PIVENS trial (that assessed the efficacy of pioglitazone or vitamin E versus placebo in 247 adult patients with biopsy proven NASH) [106] showed a significantly higher proportion of PNPLA 3 GG homozygosity among patients who did not improve fibrosis under treatment in comparison with those who obtained an improvement in fibrosis (31% versus 16% respectively, $p = 0.03$) [107].

In this line, a prospective study conducted on 261 patients with biopsy proven NASH treated with lifestyle changes for 1 year, and underwent to a second liver biopsy at the end of follow up, elaborated the NASHRES score to predict the likelihood of NASH disappearance [108]. It includes the extent of weight loss, the achievement of normal ALT levels, diabetes and baseline NAS at histology. AUCs of this model were 0.95 and 0.94 in the derivation and validation group, but the main limitation of this score is linked to the need of a baseline liver biopsy. The same authors elaborated another algorithm in the same cohort of patients to predict the impact of 1-year lifestyle changes on fibrosis improvement. This model was composed by changes in HbA1c, platelet count, NFS, ALT normalization and it has been showed to more accurate than traditional non-invasive scores alone (like NFS, FIB-4 or APRI) [109].

The potential role of serum CK-18 fragments as predictor of response to pharmacological treatment was evaluated in a post-hoc analysis of two randomized controlled trials, particularly PIVENS [106] and TONIC (a trial that assessed metformin, vitamin E or placebo in 173 patients <17 years with biopsy proven NASH) [110]. A significant decrease in CK-18 serum levels was observed in both adult and pediatric patients who had an improvement in liver histology in comparison with those without histologic improvement, independently of the treatment. However, decrease of ALT levels performed better than CK-18 in the correct identification of adult patients with histologic improvement [111].

Among the different drugs tested for the treatment of NASH, obeticholic acid (OCA), a potent and selective farnesoid X receptor (FXR) agonist, showed promising results in the FLINT trial [112], a phase IIb trial that showed a ≥2-point improvement of NAS without worsening of fibrosis in 45% of OCA-treated patients compared with 21% of placebo treated patients ($P = 0.0002$). OCA was further assessed in the ongoing REGENERATE study [113], the first phase III trial in NASH, whose prespecified interim analysis has been presented during the

last International Liver Congress, showing the OCA efficacy in ≥1-point fibrosis improvement without worsening of NASH. A recent secondary analysis of the data collected in FLINT trial aimed to identify the clinical variables, at baseline or during treatment, associated with histologic response to OCA [114]. Authors showed that baseline NAS score, triglycerides levels, INR, AST levels and a decrease in ALT levels at 24-week of treatment were significantly associated with histologic response. After the integration of these variables in a model to predict the likelihood of the achievement of histologic improvement, authors showed an AUC of 0.83, suggesting a potential usefulness of this model in the identification of patients who could obtain more benefit from OCA treatment and in the patient selection for clinical trials. Finally, another post-hoc analysis of FLINT trial evaluated the relationship between longitudinal assessment of non-invasive biomarkers (particularly, FIB-4, NFS and APRI) and fibrosis improvement [115]. Interestingly, the positive effect of OCA treatment on liver histology was showed to be correlated with improvements in APRI and FIB-4, regardless of baseline disease severity, with a higher sensitivity than NFS in the ability to predict later fibrosis stage improvements. These results appear to be relevant because they represent a proof of concept that fibrosis improvement induced by pharmacological treatments, i.e. OCA, is associated with improvement in non-invasive biomarkers. Notably, the evaluation of the relationship between OCA effects and non-invasive biomarkers is currently underway also in REGENERATE trial.

In conclusion, these data provided preliminary evidences regarding the ability of non-invasive biomarkers in prediction of histological changes in patients with NAFLD, although further studies are needed before they can be used in the clinical practice to modulate therapeutic strategies and in the field of the pharmaceutical research to better design future clinical trials.

Conclusions

NAFLD and NASH represent a growing public health problem and non-invasive biomarkers are needed for a correct management of these patients. We reviewed data and characteristics of the main available non-invasive tools: an acceptable accuracy in the identification of patients with severe liver fibrosis was showed, but data on NASH detection, on stratification of the severity of liver fibrosis and on prediction of long-term outcomes need to be implemented and further studied in the future. Finally, the next availability of effective pharmaceutical treatments for active NASH should lead to the development of *ad hoc* non-invasive biomarkers able to predict accurately response to treatment.

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Chapter 7
Non-invasive Assessment of Non-alcoholic Fatty Liver Disease: Ultrasound and Transient Elastography

Tao Wan and Annalisa Berzigotti

Introduction

As discussed in other chapters of this book, non-alcoholic fatty liver disease (NAFLD) is very prevalent in the general population as a consequence of the epidemics of obesity and metabolic syndrome and has become the main referral question to hepatologists [1, 2]. Fatty liver can occur concomitant to other chronic liver diseases (CLD), either as a consequence of comorbidities (e.g. metabolic syndrome) or due to steatogenic effects of the underlying liver disease etiology (e.g. alcohol; HCV), and contributes to worsening the CLD prognosis [3], and is a co-factor of liver fibrosis progression in HCV [4]. In viral hepatitis patients, it accelerates the onset of clinical events and reduces the likelihood of achieving sustained virological response with interferon-based therapies [5, 6]. In the surgical setting, NAFLD complicates the recovery from hepatic resection and increases the risk of graft failure after liver transplantation [7, 8]. NAFLD is also an independent predictor of serious diseases in the general population (solid neoplasias; cardiovascular events; mortality from any cause).

This data underlines the need of a reliable diagnostic method for liver fat content, able to provide data in several different clinical settings. Liver biopsy is still considered the gold-standard method not only to diagnose the presence and severity of steatosis,

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but also to identify the aggressive form of NALFD (non-alcoholic steatohepatitis, NASH), providing data on the grade of inflammation, presence of ballooning and stage of fibrosis. However, this method is invasive and might cause complications (even if rarely), it is costly and cannot be used as a screening method to point out the presence of steatosis in the general population. Non-invasive, inexpensive and accurate methods are needed to (a) diagnose NAFLD, (b) quantify the degree and possibly kind of fat accumulation (micro/macrovesicular) in the liver and (c) assess the presence and severity of NASH to better stratify the prognosis of NAFLD patients.

The ideal diagnostic method in this field should be quantitative in order to provide intra-subject comparison in the follow-up (e.g. in response to life-style changes or drug therapy). An additional ideal characteristic is that it should provide results in real-time (point-of-care diagnostic method) to shorten the time to medical decision.

The most commonly used imaging methods used for NAFLD and NASH are ultrasound and ultrasound elastography, which fulfill some of the characteristics required from ideal tests.

Ultrasound in NAFLD

Rationale and US Signs of Steatosis

Thanks to its low cost, safety, wide availability and repeatability, US is considered the first line imaging technique to screen for liver steatosis. Fat content changes the acoustic properties of the liver tissue, by generating echo interfaces. The typical "bright" liver on US is the result of increased scattering/backscattering and absorption on insonation of fat [9, 10].

On conventional B-mode (grayscale) ultrasound (US) the following signs are diagnostic of steatosis: (a) diffuse hyperechogenity of the liver ("bright liver") better seen by comparing the liver echogenicity to that of the kidney cortex or of the spleen (both usually mildly hypoechogenic in comparison to the healthy liver parenchyma); (b) tightly packed echoes, (c) reduction of ultrasound amplitude with beam attenuation in depth and (d) reduction of the echogenicity of the walls of the portal veins and/or blurring of the walls of the hepatic veins [11]. Most of these features can be used by non-experienced operators to diagnose NAFLD at bedside US examination with very good results [12].

Taking advantage of the above mentioned US signs, it has been proposed that US can classify steatosis in a semi-quantitative way, able to partially reflect the underlying histological amount of fat content [13]. Accordingly, steatosis on US is defined as *mild* when echogenicity of the liver is minimally increased, as *moderate* when posterior beam attenuation is absent or limited to posterior segments of the right lobe of the liver (diaphragm visualization is impaired) and the echogenicity of the walls of the portal vessels is reduced; while *severe* steatosis is defined when posterior beam attenuation impairs the visualization of anterior segments of the

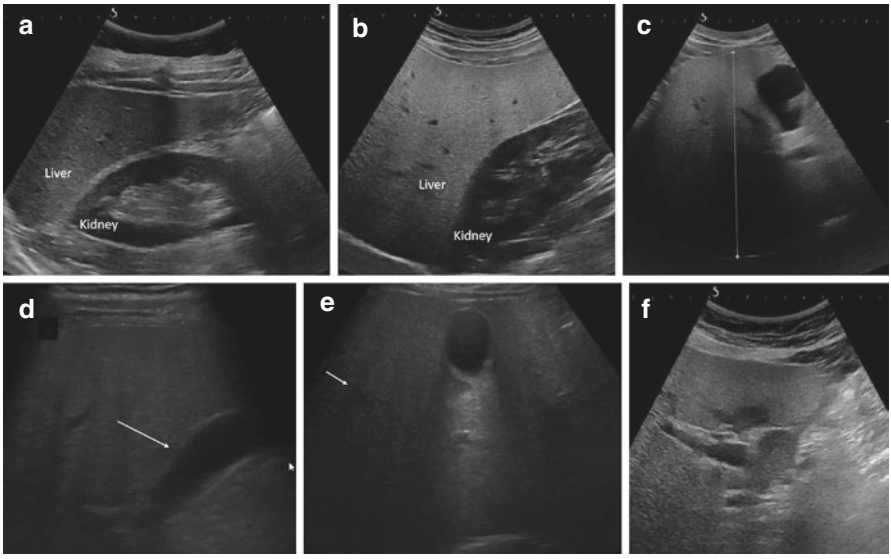

Fig. 7.1 Typical aspect of liver steatosis on ultrasound. Panel (**a**): Mild steatosis: diffuse hyperechogenic aspect of the liver with slightly increased liver/kidney echogenicity ratio. The intrahepatic vessels are well visible. Panel (**b**): Moderate steatosis: diffuse and homogeneous hyperechogenic "bright" aspect. Notice the clearly increased liver/kidney echogenicity ratio. Panel (**c**): Severe steatosis; enlarged liver with attenuation of the ultrasound beam that exceeds the posterior half of the right lobe. Panel (**d**): Steatosis with blurring of the gallbladder wall (arrow). Panel (**e**): Steatosis with blurring of the intrahepatic vessels (arrow). Panel (**f**): Area of focal sparing (arrow) can be identified as a zone of normal echogenicity within a bright liver. Most common sites include perivesicular and periportal location (such as in the present case)

liver and blurring of intrahepatic vessels is observed as well [13]. Figure 7.1 shows examples of these typical aspects.

Based on these signs, numerical scores can be calculated, and relate to the severity of the features of metabolic syndrome and of the histology grade of steatosis [14–16].

While the majority of patients with steatosis show a homogenous distribution of fat in the liver, 15–20% of cases show an *atypical*, non-uniform distribution. Zonal steatosis or focal fatty changes (FFC) refer to a bright aspect of a lobe, segment or smaller areas of the liver (most often located at the porta hepatis, adjacent to the falciform ligament and/or to the gallbladder fossa). These areas show a typical geographical shape and do not compress or infiltrate the liver vessels, which allows differentiating them from solid focal lesions. Rarely FFC can be multifocal and rounded in shape (Fig. 7.2, Panel a); in these cases, the differential diagnosis includes haemangiomas, adenomas and hyperecoic metastases and careful observation of accessory signs (lack of mass effect on adjacent tissue and vessels by FFC) is key to the final diagnosis. In difficult cases contrast-enhanced ultrasound (CEUS) can be used, since FCC show the same perfusion as the remaining parenchyma (Fig. 7.2, Panel b). Occasionally, more complex zonal or segmental steatosis can be

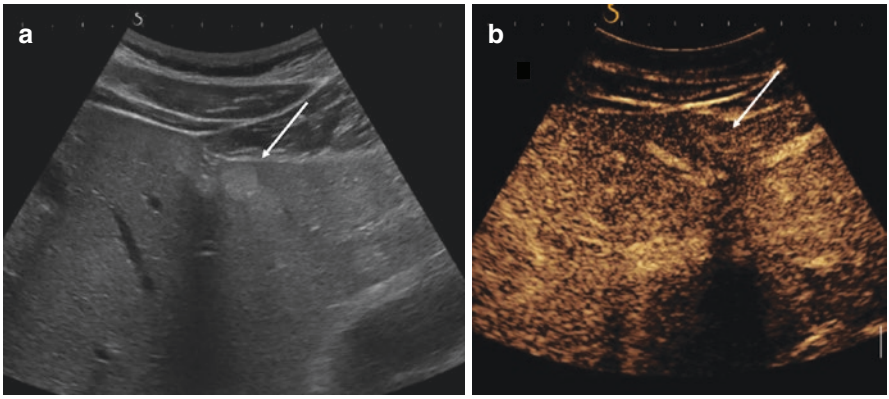

Fig. 7.2 Atypical aspects of steatosis on ultrasound. Panel (**a**): focal fatty changes (FFC) can be multifocal and rounded in shape such in this case (arrow). Differential diagnosis with metastasis or angiomas is difficult, and a contrast-enhanced imaging technique is often needed (CEUS, CT or MR). Panel (**b**): In this case, CEUS was performed; the area showed ISO-enhanced in comparison to the rest of the parenchyma in the arterial, portal and late phase, confirming the diagnosis of FFC

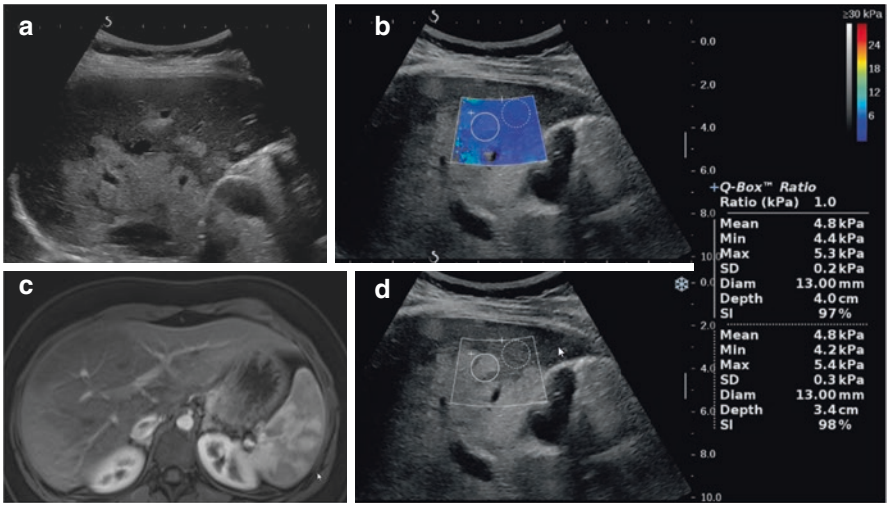

Fig. 7.3 Atypical aspects of steatosis on ultrasound. Panel (**a**): very large focal fatty changes (FFC) (arrow) in a young, asymptomatic female patient. Notice the geographic margins and the lack of compression of the intrahepatic vessels. Panels (**b** and **d**): 2D-SWE was used within the hyperechogenic area and in the normal liver parenchyma, and showed identical results, suggesting FFC. Panel (**c**): Magnetic resonance excluded solid lesions and confirmed the diagnosis of large FFC

seen (Fig. 7.3, Panel a); ultrasound elastography can provide useful data differentiating zonal steatosis from solid focal liver lesions in these cases, showing identical values of stiffness within the different parts of the parenchyma (Fig. 7.3, Panels b and d). Magnetic resonance imaging (MRI) is the method of choice to achieve a definite and final diagnosis (Fig. 7.3, Panel c).

Within a bright liver, hypoechogenic areas are often seen. These represent areas of different concentration of steatosis and are referred as *focal sparing* (Fig. 7.1, Panel f), and should not be confounded with solid focal liver lesions. They mostly appear in the locations similar to that described above for FFC, and they also usually show a geographic pattern. Both aspects might be due to differences in perfusion in these areas, leading to a different accumulation of fat and/or a different size in the fat vacuoles. In case of diagnostic uncertainty, contrast-enhanced ultrasound (CEUS) is useful, showing the same uniform enhancement as the other parts of the liver along arterial, portal and late phase.

Accuracy of US for the Detection of Steatosis

In the reported studies taking biopsy as a reference standard, sensitivity for steatosis ranges 60–100% and specificity 77–95% [17]. However, US technology changed much over the last 10 years, and the quality of B-mode echo signal improved. In a recent meta-analysis of 34 studies in 2815 patients with liver biopsy as a reference standard, the pooled sensitivity and specificity of US to differentiate between no steatosis and moderate/severe steatosis was respectively 85% and 93% [18]. Consequently, the sensitivity of US machines for steatosis increased over time, and while with old devices both steatosis and fibrosis were reported to be linked to an hyperechoic aspect of liver parenchyma, nowadays increased liver echogenicity with typical aspect (diffuse, smooth and tightly packed echoes) can be reliably considered a feature of steatosis [19]. In a more recent study, US diagnosed presence of any steatosis with a high sensitivity and positive predictive value (87%); its global accuracy was 78% in this study [20].

It should be taken into account that as any qualitative/semi-quantitative technique, the diagnosis of steatosis by US is straight-forward in moderate and severe cases, while very mild steatosis can be difficult to detect. In a prospective study, US had 90% sensitivity in detecting steatosis involving ≥20% of hepatocytes [11], while its became less sensitive for steatosis <20%. From a practical point of view, if US reports the presence of steatosis, the examined subject has likely a steatosis of at least 20% of hepatocytes. On the other hand, a normal US scan cannot exclude a steatosis <20%. Hence, in case of high clinical suspicion of NAFLD/NASH in subjects with negative US findings further, more sensitive tests should be offered (e.g. PDFF using MR, or MR spectroscopy).

Limitations of US for steatosis detection include a moderate inter-observer agreement, particularly for mild steatosis and in non-expert hands [21], and reduced applicability in morbidly obese patients [22].

False positive results are not frequent in adult patients but other diffuse liver diseases such as glycogen storage disease, can inducing bright liver and be a source of false positive US results [23].

The major limitation of ultrasound is related to its limited ability to differentiate patients with simple NAFLD from those with NASH [17] (see next paragraph), unless cirrhosis is already established [24, 25].

US and US-Based Scores to Predict the Presence of NASH

Diagnosing those patients with NAFLD who have steatohepatitis (NASH) is key to stratify the risk of fibrosis progression, and to adapt the follow-up and therapy plan ranging from lifestyle changes to standard and novel pharmacological treatments [26].

Despite it is usually assumed that US cannot differentiate NAFLD from NASH, several studies have suggested that US techniques may actually help in identifying NASH patients [15, 27–30], taking advantage from the combination of different US features of fatty liver. Proposed US parameters combinations are described in Table 7.1. Interestingly, some US features seem to reflect the inflammatory and

Table 7.1 Combinations of US parameters to predict the presence of NASH in patients with fatty liver

	Parameters included in the score	How the score is obtained	Accuracy
Ultrasound Fatty Liver Index (US-FLI) [15]	1. Liver brighter than kidney 2. Posterior attenuation of US beam 3. Vessel blurring 4. Difficult visualization of the gallbladder walls 5. Difficult visualization of the diaphragm 6. Areas of focal sparing	Mild/moderate bright liver: 2 points Severe bright liver: 3 points Each of the other features is given an additional point if present	Score ≥ 4 predicts NASH: AUROC 0.796 (NPV 94%, Sens 46%)
Modified Fatty Score (MFS) [28]	1. Parenchymal echogenicity 2. Far gain attenuation 3. Gallbladder (GB) wall blurring 4. Portal vein (PV) wall blurring 5. Hepatic vein (HV) blurring Each parameter is scored 0 to 2; the sum is the Fatty Score	Modified Fatty Score is: 0 if Fatty Score < 7 1 if Fatty Score ≥ 7 and parenchymal ecogenicity + GB blurring is <3 2 if Fatty Score ≥ 7 and parenchymal ecogenicity + GB blurring is ≥3	MFS cut-off ≥2: 72% Sens. and 86% Spec. for NASH
Zardi's score [29]	1. Attenuation of the echo amplitude 2. Presence or absence of focal fat sparing 3. Splenic diameter	1. Attenuation: 0 if absent, 1 if mild, 2 if severe 2. 0 if absent, 1 if present 3. 0 if <120 mm; 1 if 120–140 mm, 3 if >140 mm	Score > 5: poor performance for NASH (Sens 74%; Spec. 66%) Attenuation + focal sparing discriminated NASH from steatosis (Sens 92%, Spec 75%)

hepatocyte ballooning components of NASH rather than fibrosis, and as such these parameters could be complementary to the information provided by elastography.

The Ultrasound Fatty Liver Index (US-FLI) (based on a scoring system including the intensity of liver/kidney contrast, posterior attenuation of ultrasound beam, vessel blurring, difficult visualization of gallbladder wall, difficult visualization of the diaphragm and areas of focal sparing) was an independent predictor of NASH (OR 2.236; P = 0.007) and ruled-out severe NASH if <4 in one study using liver biopsy as a gold standard [15].

In a small study by Zardi et al., combining attenuation of the echo amplitude and presence of focal fat sparing, was able to differentiate NASH from steatosis with 92% sensitivity and 75% specificity [29].

Finally, the Modified Fatty Score (MFS) was able to predict NASH with 72% sensitivity and 86% specificity in a morbidly obese patients undergoing bariatric surgery [28]. Only the MFS was correlated with liver fibrosis, while the US-FLI and Zardi's score did not. These results are not unexpected considering that liver echogenicity reflects steatosis but not fibrosis [19].

In the detection of NASH, US-FLI showed higher sensitivity but lower specificity than the MFS and Zardi's score [15, 28, 29].

Spleen size (longitudinal diameter) might be also related with the potential presence of NASH and a spleen size >11.6 cm was able to discriminate between patients with simple steatosis and NASH with a sensitivity of 88% and a specificity of 95% in one study [30]. In addition, splenomegaly suggests the presence of portal hypertension in patients with cirrhosis due to NAFLD/NASH [31].

US for Liver Cirrhosis and Portal Hypertension in NAFLD

Liver fibrosis is a key factor in determining the outcome of all chronic liver diseases, including NAFLD since it is associated with the development of portal hypertension, cirrhosis and liver failure. B-mode US is inaccurate in detecting fibrosis in pre-cirrhotic stages. Even if quantitative Doppler can improve the US diagnosis of cirrhosis, the real game-changer has been the development of US elastography, allowing to measure liver stiffness (a good marker of liver fibrosis) [32]. Before elastography was available, some authors assessed whether Doppler signs improve the standard US diagnosis of NASH, and whether it allows a more accurate follow-up of this condition.

Moderate and severe steatosis are often associated to biphasic and monophasic flow, indicating a dampening of the normal spectral Doppler waveform in the hepatic veins [33–35]. Interestingly, improvement of fat content can lead to normalization of hepatic veins flow pattern [16]. In NAFLD and in particular in NASH, blood flow velocity in the portal vein is often decreased below the normal values [16, 35, 36], and the hepatic artery resistive index is increased [16, 33]. Improvement in these parameters has been described in patients undergoing effective treatment of NAFLD.

As for cirrhosis and portal hypertension, findings on B-mode in NAFLD/NASH are similar to those described in other etiology, namely: nodular liver surface, coarse

echopattern of the parenchyma, blunted liver edge, liver morphology changes (e.g. right lobe atrophy; enlarged caudate lobe), splenomegaly, increased in portal vein diameter with reduction in the respiratory variation; presence of porto-collateral circulation and ascites [32, 37]. In the experience of the authors, due to the concomitant fat content, the nodularity of liver surface can be blunt in patients with compensated cirrhosis due to NAFLD/NASH, and the presence of this sign needs to be carefully assessed using high-frequency linear probes on the left liver lobe. The use of multimodal ultrasound including liver elastography (see specific paragraph below) is of particular importance in patients with indeterminate findings on ultrasound, and can lead to early diagnosis of severe fibrosis and cirrhosis.

No US data specific to NAFLD/NASH have been published with respect to the use of ultrasound contrast agents transit time.

Novel Tools for Steatosis Quantification on US Images

As previously discussed, conventional US does not allow for exact quantification of the amount of hepatic fat. Based on the possibility of digitalizing the images obtained by US there have been several attempts to use computerized mathematical analysis and quantification of fat through mathematical modelling. These are summarized in detail elsewhere [38] and in the present book chapter we will only discuss those methods closer to clinical practice.

The Sonographic Hepatorenal Index (SHRI) is a ratio calculated by informatic means between the mean brightness level of the liver and the right kidney using selected region of interest pixels. Using SHRI in 111 consecutive patients with liver disease of various etiology (including NAFLD) steatosis over 5% could be detected with 100% sensitivity and 91% specificity by a cut-off of 1.49 hat SHRI [39]. In another study in 42 NAFLD patients and 40 healthy controls, a lower cut-off (1.24) with a sensitivity and a specificity of 93% has been proposed [40]. In this study, SHRI, which is a numeric parameter, increased as the amount of steatosis on histology increased. Other studies support that SHRI is a feasible and simple method to quantify steatosis on US [41, 42], and in a recent study, SHRI showed a strong correlation (Spearman's coefficient = 0.89, $p < 0.001$) with fat content on H-MRS (H-Magnetic Resonance Spectroscopy) [43]. In this study, 100% sensitive cut-off points for 5%, 25% and 50% fat content using SHRU were respectively 1.21, 1.28 and 2.15; specificity was >70% in all [43]. However, more recent studies reported substantially higher cut-offs [44, 45] and despite this index seems to be close to clinical validation, a better standardization of the analysis technique and cut-offs is needed.

In the attempt of making standardization possible, Xia et al. [42] reported that hepatic/renal echo-intensity ratio and ultrasound hepatic echo-intensity attenuation rate obtained from standard US images and standardized using a tissue-mimicking phantom before analysis well correlated with the liver fat content assessed by magnetic resonance spectroscopy ($r = 0.884$ and $r = 0.711$, $P < 0.001$, respectively).

These parameters were reproducible across machines and operators, and could be easily implemented in routine machines if validated.

Other methods, studied in a rabbit [46] and in rat [10] models of NAFLD, take advantage of the analysis of ultrasound backscatter (the first by the directly estimating effective scatterer diameter and effective acoustic concentration; the second by assessing the statistical distribution on parametric imaging-Nagakami parameter), and showed an excellent correlation with the fat concentration in the liver, suggesting that these quantification methods may be applied in the future for the analysis of images of human steatosis [10, 46].

Very recently, the Ultrasound Guided Attenuation Parameter (UGAP) has been proposed. This measures the attenuation coefficient (AC) (dB/cm/MHz) of B mode ultrasonic signal with general ultrasonography. UGAP showed significant correlations were found between attenuation coefficient and percentage steatosis, CAP values (measured by Vibration Controlled Transient Elastography) and liver-to-spleen CT attenuation ratio ($p < 0.001$). The UGAP AUROC was 0.900, 0.953 and 0.953 respectively for any steatosis, moderate steatosis and severe steatosis, being UGAP significantly better than CAP [47].

Sound speed estimation (SSE) is another suggested US based method which could provide data on presence and grade of steatosis. In a recent study in 100 patients (50 in the training cohort and 50 in the validation cohort), [48] assessed SSE for the detection, quantification, and grading of hepatic steatosis using magnetic resonance (MR) proton density fat fraction (PDFF) as reference standard.

The SSE threshold of $\leq$1.537 mm/μs had a sensitivity of 80% and a specificity of 85.7% to diagnose steatosis (S1–S3) in the training cohort and a robust correlation between MR-PDFF and the US fat index was found both for the training ($R2 = 0.73$) and the validation cohort ($R2 = 0.76$). SSE is therefore a promising method to quantify fat content on standard US examinations.

Controlled Attenuation Parameter (CAP) for Steatosis

Ultrasound waves progressively loose amplitude while crossing tissues; this physical phenomenon is termed "attenuation", and is described by a complex equation, which includes ultrasound beam frequency, length of propagation in the tissue, and the attenuation coefficient specific of a given medium/tissue among others. Since fat affects the physical properties of the liver tissue, and the ultrasound wave propagation through the tissue, a method able to measure the degree of ultrasound attenuation would reflect fat content. Fatty infiltration increases US attenuation in a proportion-dependent manner, so that if frequency of emission and tissue length are maintained fixed, it can be measured, and this is the principle used for Controlled attenuation parameter (CAP) measurements.

CAP is performed on vibration-controlled transient elastography (VCTE) implemented on FibroScan® (Echosens, Paris, France) [49], at the standardized frequency emission of 3.5 MHz with the M probe and 2.5 MHz with the XL probe (central

frequency of emission). CAP software output is a numerical value, and is operator-independent. Values range from 100 to 400 dB/m, and the final result is the median value of 10 valid measurements [49]. It is measured simultaneously to LS measurement, and as such, it suffers from the same limitations. Using the M probe, obesity (defined as a BMI >30 kg/m^2) is the main cause of CAP measurement failure, which has been reported in up to 7.7% of cases [50]. The recent implementation of CAP on the XL probe seem to have overcome this major limitation.

As for CAP ability to quantify steatosis, most data has been obtained in patients with CLD of different etiologies [49–60]. In the initial series, CAP showed a good correlation with fat content on liver biopsy [49, 50, 54, 55, 61–66], and the strength of the association was not influenced by the presence of fibrosis or cirrhosis in most studies. In the largest available prospective study, de Ledinghen et al. [50] showed that CAP had AUROCs of 0.79, 0.84 and 0.84 for histological steatosis >10 (S1), >33 (S2) and >66% (S3), respectively, and performed better than serum indices of steatosis. A recent individual data meta-analysis in 2735 patients studied with M probe, 20% of which with NAFLD, showed AUROCs of 0.82, 0.86 and 0.88 for S1, S2 and S3 respectively [67]. CAP values were influenced by the presence of diabetes, BMI and NAFLD.

As for the best cut-offs to be used, they are not yet completely defined. Values >215 dB allowed the detection of fatty infiltration ≥10% of hepatocytes with a sensitivity over 90% in two recent studies in CLD and NAFLD [50, 65]. CAP values below this threshold might be therefore considered normal and can be used to exclude steatosis. The meta-analysis by Karlas et al. [67] determined a best cut-off of 248 dB/m for S1, 268 dB/m for S2 and 280 dB/m for S3. The meta-analysis also suggests that even if CAP is an excellent method to identify steatosis and severe steatosis, it seems less accurate to differentiate adjacent grades of steatosis. It should be underlined that in most studies CAP values >300 dB were consistently associated to severe steatosis and more severe features of metabolic syndrome in the largest study published so far [50].

In patients without known chronic liver disease (general population), CAP correlates with the number of features of metabolic syndrome [50], likely reflecting liver steatosis in this setting [68, 69]. Insulin resistance and increased uric acid, as well as an increase LSM (>7 kPa) remained independently associated with CAP in one study, suggesting again that patients with risk factors for NASH have higher CAP values, likely reflecting higher grade of steatosis [50]. A high prevalence of increased CAP values have been found in patients with type 2 diabetes mellitus [70] and in obese children [71], who also showed increase LSM values.

The performance of CAP in the NAFLD/NASH domain has been assessed by a limited number of studies, which are summarized in Table 7.2. Interestingly, in 50 biopsy-proven NAFLD subjects CAP had a diagnostic ability in quantifying hepatic steatosis comparable to that of proton magnetic resonance spectroscopy (H-MRS) [65].

Large prospective studies are required to validate the available CAP cut-offs of M probe and/or to develop new cut-off values using the XL probe, since the two existing studies show conflicting results [72–74]. Recently, a study performed in

Table 7.2 Diagnostic performance of CAP compared with liver biopsy for the detection of steatosis taking biopsy as a reference standard

Author and ref	Patients (n)	Probe	Steatosis grade	Cut-off (dB/m)	AUROC
Sasso et al. [49] (2010)	115	M	S ≥ 1	238	0.91
			S ≥ 2	259	0.95
			S3	293	0.89
Myers et al. [54] (2012)	153	M	S ≥ 1	289	0.79
			S ≥ 2	288	0.76
			S3	283	0.70
de Lédinghen et al. [55] (2012)	112	M	S ≥ 1	266	0.84
			S ≥ 2	311	0.86
			S3	318	0.93
Kumar et al. [56] (2013)	317	M	S ≥ 1	214	0.68
			S ≥ 2	255	0.79
			S3	305	0.91
Shen et al. [57] (2014)	189	M	S ≥ 1	253	0.92
			S ≥ 2	285	0.92
			S3	310	0.88
Chan et al. [58] (2014)	101	M	S ≥ 1	263	0.97
			S ≥ 2	263	0.86
			S3	281	0.75
Karlas et al. [65] (2014)	50	M	S ≥ 1	233	0.93
			S ≥ 2	268	0.94
			S3	301	0.82
Lupsor-Platon et al. [117] (2015)	201	M	S ≥ 1	260	0.81
			S ≥ 2	285	0.82
			S3	294	0.83
de Lédinghen et al. [118] (2016)	261	M	S ≥ 2	310	0.80
			S3	311	0.66
Imajo et al. [51] (2016)	127	M	S ≥ 1	236	0.88
			S ≥ 2	270	0.73
			S3	302	0.70
Park et al. [119] (2017)	104	M	S ≥ 1	261	0.85
		XL	S ≥ 2	305	0.70
			S3	312	0.73
Runge et al. [120] (2017)	55	M	S ≥ 1	260	0.77
			S ≥ 2	296	0.78
			S3	334	0.78
Chan et al. [73] (2017)	57	M	S ≥ 1	260	0.94
		XL	S ≥ 2	266	0.80
			S3	267	0.69
Naveau et al. [121] (2017)	194	XL	S ≥ 1	308	0.85
			S ≥ 2	335	0.59
			S3	341	0.39

(continued)

Table 7.2 (continued)

Author and ref	Patients (n)	Probe	Steatosis grade	Cut-off (dB/m)	AUROC
Siddiqi et al. [122] (2018)	393	M and XL	S ≥ 1	285	0.76
			S ≥ 2	311	0.70
			S3	306	0.58
Eddows et al. [123] (2019)	380	M and XL	S ≥ 1	302	0.87
			S ≥ 2	331	0.77
			S3	337	0.70

the Unites States in over 350 obese NAFLD patients [75] reported a 96% positive predictive value for the cut-off of 263 dB/m using the XL probe, but the results have not been validated so far.

As for whether US or CAP should be used for screening for NAFLD, a head-to-head comparison with ultrasound has been performed in few studies with conflicting results. In one study CAP was able to detect steatosis in patients with normal US, suggesting that it might be more accurate [69], but in another study both methods had a similar, good accuracy for the diagnosis of steatosis [68].

Fibrosis Assessment in NAFLD: Transient Elastography

Liver fibrosis is the major driver of prognosis in patients with NAFLD [1], and it is the major feature to be detected in patients with suspected NAFLD/NASH.

Transient elastography (TE) (Fibroscan®, Echosens, Paris, France) is the first, well validated non-invasive tool to quantify liver fibrosis in most chronic liver diseases [76]. It is composed by a vibratile element inducing mild amplitude, low frequency vibrations (50 Hz) and by a 5 MHz ultrasound transducer probe on the tip. Vibrations are transmitted to the liver tissue by applying the probe to the skin in an intercostal space; the transmission generates an elastic shear wave whose propagation velocity is measured by pulsed echo acquisition [77]. The harder the tissue, the faster the shear wave propagates. Despite liver stiffness is a complex property of the tissue, with several components, in a simplistic way, while the normal liver is soft, fibrosis increases liver stiffness (LS).

The time needed to explore a patient is short (<5 min) and the examination is completely pain free. The results are expressed in kilo Pascals (kPa) as the median of 10 valid measurements. Inter- and intra-observer reproducibility are >90% in expert hands [78]. It explores a relatively large area of liver parenchyma (approximately 1 cm × 4 cm), the volume of which is about 100 times that of a liver biopsy [77, 79]. The liver stiffness measurement (LSM) of a normal liver is <5.5 kPa, with values ranging up to 75 kPa in disease [80].

A large number of studies have explored the use of VCTE in patients with NAFLD, with data derived from both Asian and Western series, and both in adult and pediatric cohorts; those which are most relevant to the present chapter are listed in Table 7.3. In NAFLD, LSM by VCTE has been shown to have good diagnostic

Table 7.3 Performance of transient elastography (M and XL probe) for the staging of liver fibrosis in patients with NAFLD in published series taking biopsy as a reference standard

Author and ref.	Patients (n)	Probe	Fibrosis stage	Cut-off (kPa)	AUROC
Nobili et al. [124] (2008)	50	M	F ≥ 2	7.4	0.99
			F ≥ 3	10.2	1.00
			F4	NA	NA
Yoneda et al. [81] (2008)	97	M	F ≥ 2	6.6	0.86
			F ≥ 3	9.8	0.90
			F4	17.5	0.99
Wong et al. [83] (2010)	246	M	F ≥ 2	7.0	0.84
			F ≥ 3	8.7	0.93
			F4	10.3	0.95
Lupsor et al. [125] (2010)	65	M	F ≥ 2	6.8	0.79
			F ≥ 3	10.4	0.98
			F4	NA	NA
Petta et al. [94] (2011)	146	M	F ≥ 2	7.2	0.79
			F ≥ 3	8.2	0.87
			F4	NA	NA
Gaia et al. [126] (2011)	72	M	F ≥ 2	7.0	0.80
			F ≥ 3	8.0	0.75
			F4	10.5	0.94
Myers et al. [98] (2012)	75	M	F ≥ 2	7.8	0.86
			F ≥ 3	NA	0.87
			F4	22.3	0.88
Wong et al. [96] (2012)	129	M	F ≥ 2	7.0	0.83
			F ≥ 3	8.7	0.87
			F4	10.3	0.89
Kumar et al. [127] (2013)	205	M	F ≥ 2	7.0	0.85
			F ≥ 3	9.0	0.94
			F4	11.8	0.96
Petta et al. [113] (2015)	179	M	F ≥ 3	7.9/9.6	0.86
Pathik et al. [74] (2015)	110	M	F ≥ 2	9.1	NA
			F ≥ 3	12.0	NA
			F4	20.0	0.91
Imajo et al. [51] (2016)	127	M	F ≥ 2	11.0	0.82
			F ≥ 3	11.4	0.88
			F4	14.0	0.92
Cassinotto et al. [108] (2016)	291	M	F ≥ 2	6.2	0.82
			F ≥ 3	8.2	0.86
			F4	9.5	0.87
Tapper et al. [128] (2016)	164	M	F ≥ 3	9.9	0.93
Boursier et al. [129] (2016)	452	M	F ≥ 3	8.7	0.83
Petta et al. [130] (2017)	324	M	F ≥ 3	10.1	0.86
Park et al. [119] (2017)	104	M and XL	F ≥ 3	7.3	0.80
			F4	6.9	0.69

(continued)

Table 7.3 (continued)

Author and ref.	Patients (n)	Probe	Fibrosis stage	Cut-off (kPa)	AUROC
Chen et al. [131] (2017)	111	M and XL	F ≥ 3	7.6	0.87
			F4	14.6	0.92
Petta et al. [87] (2017)	761	M	F ≥ 3	7.9/9.6	0.86
Siddiqi et al. [122] (2018)	393	XL	F ≥ 3	8.6	0.83
			F4	13.1	0.93
Eddowes et al. [123] (2019)	373	M and XL	F ≥ 2	8.2	0.77
			F ≥ 3	9.7	0.80
			F4	13.6	0.89

accuracy for the presence of fibrosis, with an AUROC of 0.927 for ≥F1 fibrosis [81, 82], and excellent AUROC of 0.93 (95% CI 0.89–0.096) has been reported for advanced fibrosis (≥F3) and cirrhosis, with a negative predictive value of 90% in ruling out cirrhosis when using a cut-off of LSM 7.9 kPa [83].

These results have been confirmed by two meta-analysis [84, 85]. In the most recent, including 19 studies and 2495 NAFLD patients [85], summary AUROCs for advanced fibrosis and cirrhosis with M probe was respectively 0.87 and 0.92, and with XL probe 0.86 and 0.94, respectively. The choice of the best cut-offs to use still faces difficulties; proposed ranges are 6.6–7.8, 7.1–10.4 and 10.3–22.3 kPa corresponding to stages F2, F3 and F4 respectively [86].

Importantly, as for other etiologies, VCTE is not robust to differentiate between adjacent stages of fibrosis. Despite this, VCTE seems to have the features of an excellent screening test to rule-out fibrosis and identify advanced fibrosis and cirrhosis.

The limitations of VCTE are well known, and also apply to patients with NAFLD. In some studies high grades of steatosis (either on US or on CAP) [87] and high necroinflammatory activity led to increase of LSM independent of fibrosis, so leading to overestimation of fibrosis [88–91]. Petta et al. [87] suggests to use a higher cut-off value for fibrosis in patients with NAFLD and high steatosis on CAP to avoid excessive false positive results of VCTE (and likely excess of not necessary liver biopsies) in this population.

The reproducibility of measurement is lower in patients with NAFLD and obesity [78]. Before the XL probe was made available, VCTE failed in up to 25% of attempted studies in obese patients [92, 93], who also often showed over- or underestimation of liver fibrosis [94, 95].

XL probe, which has a lower frequency and as such a higher penetration capability, has been designed to overcome the limitations of M probe for liver stiffness assessment using VCTE [96]. Despite the feasibility of the test in obesity improved markedly, and LSM has a lower discordance with histology findings using XL probe in this population (ranging 9–11% [96, 97]), still 3–7% show failure to measure even with XL probe [98].

Whether the values obtained by XL probe are similar or lower as compared to M probe, conflicting results have been reported. Most studies reported a median

LSM by the XL probe 1–1.2 kPa lower than that of M probe for the same stage of fibrosis [96, 99]. However, this might be due to the fact that only one of the probes is adequate to the specific skin-to-capsule distance of a given patient, and only the adequate probe should be used.

With the limits due to possible selection bias in tertiary centers, VCTE using XL probe showed an AUROC of 0.80, 0.85 and 0.91 for F ≥ 2, F ≥ 3 and F4, respectively; a cut-off of 7.2 kPa for advanced fibrosis (≥F3), showed 78% sensitivity and specificity, with 89% negative predictive value in an obese population with NAFLD [96]. In another study in severely obese patients [100] XL probe showed a good diagnostic performance for the detection of significant (AUROC: 0.81 ± 0.05) and advanced fibrosis (AUROC: 0.85 ± 0.04).

Data on the use of VCTE to follow-up patients with NAFLD are very scarce, and are limited to a prospective 4-year study conducted by Suzuki et al. [101].

In summary, VCTE is useful to rule-out patients with NAFLD and low risk of severe liver fibrosis, which are candidate to follow-up, and can reliably point-out patients with probable severe fibrosis or cirrhosis, deserving further tests (MRE; liver biopsy; endoscopy to screen for varices if LSM >20–25 kPa or platelet count <150 G/L) [102]. Confounders leading to high liver stiffness independent of fibrosis (cholestasis; liver congestion; meal ingestion, severe steatosis, high necroinflammatory activity) should be carefully taken into account on interpreting VCTE results in NAFLD.

Sonoelastographic Methods to Diagnose and Quantify Liver Fibrosis: Point Shear-Wave Elastography (pSWE) and bidimensional Shear-Wave Elastography (2D-SWE)

Given the strong evidence in favour of non-invasive staging of fibrosis by liver stiffness assessment, in recent years different ultrasound machines manufacturers developed novel methods able to overcome, at least in part, the limitations of TE. pSWE and 2D-SWE constitute a novel group of sonoelastography techniques based on Acoustic Radiation Force Impulse imaging (ARFI) embedded in standard ultrasound devices. They have the major advantage of allowing measurement of liver stiffness under direct dynamic ultrasound visualization, and of creating the compression impulse directly in the liver tissue (less prone to artefacts due to the thoracic wall and due to ascites). Measurements have to be made at least 1–2 cm below the liver capsule to obtain the best predictive value.

In pSWE the compression impulse in the tissue is generated by a short-duration acoustic pulse in a specific point, which generates shear waves which propagate in the tissue. The velocity of the shear waves can be measured and is proportional to the stiffness of the tissue. The region of interest is 1–2 cm and is smaller than that of VCTE. The Virtual Touch Tissue Quantification (Acuson 2000, Siemens Healthcare, Erlangen, Germany) is the best validated pSWE technique [103]. The diagnostic

accuracy of this technique for liver fibrosis assessment is similar to that of VCTE in chronic liver diseases of different etiologies [61, 104, 105], and in NAFLD [61, 106]; interestingly, its rate of technical failure is lower than that of M probe (but not XL probe) with TE in severely obese patients. In a meta-analysis of seven studies including 723 patients with NAFLD studied with this technique, significant fibrosis was detected with a pooled sensitivity of 80.2% and specificity of 85.2% [107]. In a comparative study with VCTE in 291 NAFLD who underwent liver biopsies, the two techniques showed similar and good accuracy for advanced fibrosis (≥F3: AUROC 0.87 for VCTE and 0.85 for pSWE Virtual Touch) [108]. In another meta-analysis comparing the diagnostic performance of pSWE and VCTE for significant fibrosis and cirrhosis, no difference between the two was found [109]. However, some studies have reported results contrasting with this conclusion. Ebinuma et al. [104] and Friedrich-Rust et al. [61] found a high diagnostic accuracy of pSWE in detecting significant and severe fibrosis but a poorer correlation with the different stages of fibrosis as compared with VCTE. As for the cut-offs to be used for cirrhosis, data specific to NAFLD are lacking. In a meta-analysis of 36 studies involving 3951 patients of different etiologies [110] a cutoff value of 1.87 m/s, had 84% sensitivity and 92% specificity for the diagnosis of cirrhosis.

In 2D-SWE the impulse is generated simultaneously in several points within a chosen area of the liver tissue. Different 2D-SWE algorithms are currently available on different US devices, but a sufficient amount of published data is only available for the 2D-SWE of Aixplorer (Supersonic Imagine, France). In a study including 291 patients with NAFLD [108], 2D-SWE diagnostic accuracy for advanced fibrosis and cirrhosis was respectively 89% and 88%, and the cut-offs with >90% sensitivity were 8.3 kPa and 10.5 kPa respectively; in this study, 2D-SWE was superior to VCTE for fibrosis.

In a subgroup of 172 NAFLD patients included in a meta-analysis of published data [111], the diagnostic accuracy for advanced fibrosis and cirrhosis was respectively 93% and 92%, and the best cut-offs were 9.2 kPa and 13.5 kPa respectively. As compared to VCTE, 2D-SWE showed a similar accuracy for cirrhosis and a significantly better accuracy for advanced fibrosis (12% difference).

Diagnostic Algorithm for the Rationale Use of US and Elastography in NAFLD

The best diagnostic algorithm for patients suspected of NAFLD/NASH is still matter of debate. The EASL guidelines suggest using a combination of tests, since they likely perform better than a single modality alone. However, no robust longitudinal data supporting the use of the different available algorithms to predict clinically relevant outcomes are available. In our practice, we use ultrasound and/or CAP to identify NAFLD. If they suggest steatosis in a compatible clinical setting, we use NAFLD Fibrosis Score (NFS) and VCTE [112] to assess the likelihood of liver fibrosis/NASH (Fig. 7.4).

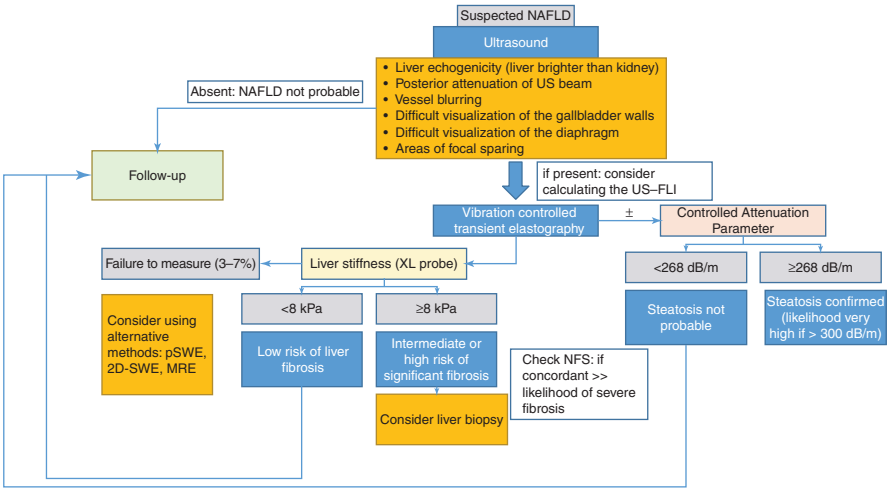

Fig. 7.4 Proposed algorithm for the assessment of suspected NAFLD using ultrasound and VCTE. As shown, ultrasound, elastography and CAP can be used together with clinical and laboratory information (NFS) to guide clinical decisions in NAFLD patients

A similar approach has been used by Petta et al. [113], who showed that the combination of NFS and VCTE are superior to other non-invasive tests (APRI, AST/ALT, BARD, FIB-4) to identify advanced fibrosis in patients with NAFLD. This strategy has not only an excellent performance, but likely also represents a cost-effective strategy [114]. When VCTE fails to provide information, we usually measure liver stiffness by 2D-SWE, which is available at our center. In selected cases with high likelihood of NASH based on the laboratory and clinical features who have bad US visualization, we chose to perform MRE and MRI, in addition to liver biopsy.

Conclusions

Liver US is still an accurate to diagnose moderate and severe steatosis; with the aid of simple qualitative and semi-quantitative assessment of the severity of fatty infiltration, US can reliably identify patients with higher probability of carrying NASH. The use of CAP may further improve the sensitivity in detecting hepatic steatosis, and the use of US, CAP and LSM as screening methods, for instance in patients with high pre-test probability of carrying NASH (i.e. those with type 2 diabetes and obesity) is desirable. In our view they seem useful and cost-effective methods allowing identifying patients requiring liver biopsy to confirm, grade and stage NASH [115, 116], and reasonably sparing liver biopsy in those patients with a lower risk profile. This approach is outlined in Fig. 7.4.

Novel technological advances already allow the measurement of liver stiffness on US devices, and multimodality will likely further expand, allowing soon the

quantification of fat content on ultrasound images. This will facilitate an even earlier diagnosis of patients with NAFLD, potentially benefitting from life-style interventions before developing NASH. Well-designed head-to-head studies comparing US and CAP in this field will become necessary.

In patients with NAFLD, liver stiffness measurement is accurate to diagnose severe fibrosis and cirrhosis, but the interpretation of the results stills suffers from the confounding effect of inflammation. A comprehensive ultrasound tool providing quantitative data in real-time on all the aspects characterizing the NAFLD/NASH spectrum (fat content, inflammation, hepatocyte ballooning and fibrosis) is still an unmet need in hepatology, awaiting for novel answers.

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Chapter 8
MR Based-Imaging Biomarkers in NAFLD/NASH

Michael Pavlides

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the Western World affecting up to a third of the adult population [1]. The disease varies in severity from accumulation of liver fat only (simple steatosis) to fat associated with inflammation (non-alcoholic steatohepatitis; NASH) and fibrosis and cirrhosis. It is now well established that patients with fibrosis are at increased risk of morbidity and mortality, while patients with simple steatosis generally have better prognosis [2, 3]. The prognostic importance of NASH remains a matter of debate [4].

The diagnostic classification of NAFLD into simple steatosis and NASH and the assessment of fibrosis relies on liver biopsy. This presents a challenge in clinical practice and in the conduct of clinical trials. In clinical practice, it is important to identify patients with high risk of morbidity/mortality from NAFLD so that they be prioritised for follow up in secondary care and for appropriate surveillance in cases of liver cirrhosis. As NAFLD is highly prevalent, liver biopsy is not practical as a diagnostic tool that needs to be applied at the level of the population, due to its, costs and invasiveness.

Liver biopsy and histological assessment of fibrosis and NASH are the only approved surrogate end points in clinical trials. Patients taking part in clinical trials therefore need to have repeated liver biopsies. Sampling errors and observer dependent variability in liver biopsy reporting means that more patients have to be recruited to achieve sufficient statistical power, while studies also suffer from high

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screening failure rates and dropouts. Presence of NASH and fibrosis are also important for inclusion into clinical trials.

Steatosis has traditionally been regarded as a "benign" feature in NAFLD that has no bearing on the progression of liver disease. This may be in part because steatosis is routinely quantified histologically as the "number of hepatocytes containing lipid droplets" which may not give an accurate estimate of the liver fat content. MRI on the other hand can quantify liver fat as a proportion (fat fraction; %). of liver tissue and is more accurate than histology [5]. In a natural history study, patients with liver fat fraction ≥15.7% as measured by MRI proton density fat fraction (PDFF) were more likely to progress their fibrosis than patients with fat fraction <15.7% were (multivariable adjusted odds ratio 6.7; 95% CI 1.01–44.1; p = 0.049) [6].

Furthermore, evidence is emerging from clinical trials where liver fat is being assessed with MRI PDFF that suggests that reduction in liver fat is associated with histologic improvements. Data suggest that a relative decrease of 30% in liver fat is associated with improvements in NASH (≥2 points reduction in the NAS score) [7–9] and steatosis on biopsy and serum markers of fibrosis and NASH activity [10–13]. Furthermore, the study of the fibroblast growth factor −19 analogue NGM282 produced a relative reduction in liver fat of 58% and 67% in those treated with 1 mg and 3 mg respectively and this was associated with improvement in fibrosis [14, 15].

In summary, there is an unmet need for non-invasive biomarkers of fibrosis as this is an important prognostic factor, for the diagnosis of NASH for inclusion in clinical trials and assessment of effectiveness and for steatosis that can be an early predictor of response to treatment. To address these areas of unmet clinical need and to reduce reliance on liver biopsy for the assessment of NAFLD in different contexts, several non-invasive techniques have been developed. These are generally divided into serum-based biomarkers (direct and indirect), ultrasound elastography based biomarkers and magnetic resonance based biomarkers, which will be the focus of this chapter. In general, simple indirect serum based markers are recommended for population screening in the community with direct serum markers [16] and transient elastography [17] reserved as a second tier of assessment. MR based biomarkers are generally reserved for cases where transient elastography fails [17].

Several MR biomarkers have been explored for several aspects of liver disease, focusing mainly on the distinction of NASH vs. non-NASH, the quantification of fibrosis, and for the monitoring of treatment response.

Magnetic Resonance Elastography

Overview

Magnetic Resonance Elastography (MRE; Resoundant, Rochester, US) is an MR technique that measures liver stiffness. Additional hardware and software is needed in order to carry out MRE and adaptations need to be made to the MR suite to accommodate these. During MRE, a plastic circular device is attached to the patient over the region of the liver. Mechanically generated shear waves are transmitted

through the circular device to the liver and their propagation is visualised using specific MR sequences (e.g. 2D-gradient recalled echo (GRE) pulse sequences). These data are then used to provide an estimate of the liver stiffness, which is mostly considered a biomarker of fibrosis. 2D-MRE is clinically available and is the most validated of the MR based biomarkers in NAFLD having been tested in approximately 700 patients. 3D-MRE is also in development and this has also been explored in the assessment of patients with NAFLD. 3D-MRE gives information additional to stiffness and early studies show that it may result in improved performance.

MRE has a low failure rate (4.3%) [18] and excellent inter-observer agreement (intraclass correlation coefficient 0.95) [19].

NASH vs. Non NASH

In a retrospective study the area under the receiver operating curve (AUROC) of 2D MRE for the diagnosis of NASH was reported as 0.93 [20] (threshold 2.74 kPa, Se 0.94, Sp 0.73, PPV 0.85, NPV 0.89; Threshold 2.90 kPa Se 0.83, Sp 0.82, PPV 0.88, NPV 0.75). However, this level of performance was not replicated in five prospective studies that reported area under the curve (AUC) ranging from 0.70 to 0.81 [21–25]. Furthermore, these studies report on the best thresholds derived on their population. There is therefore no prospective validation on the performance of predefined cut-offs. MRE does not offer any improvement in the diagnosis of NASH compared to transient elastography [22, 25].

Studies that have examined 3D MRE for the diagnosis of NASH have also reported only moderate diagnostic accuracy. In a study of 100 patients 3D MRE (60 Hz) and 3D MRE (40 Hz) had AUROC of 0.76 and 0.74 respectively, compared to 2D MRE (60 Hz) of 0.75 [23]. In patients who were undergoing bariatric surgery, the AUROC for the diagnosis of NASH was 0.73 and for the evaluation of disease activity using the NAS score was 0.82 [26].

Staging of Fibrosis in NAFLD

The performance of MRE for the assessment of fibrosis has been the subject of meta-analysis. In a meta-analysis of 5 studies including 628 patients, the mean AUC of the pooled data for the diagnosis of significant fibrosis ($F \geq 2$), advanced fibrosis (≥ 3) and cirrhosis were 0.88 (95% CI 0.83–0.92), 0.93 (0.90–0.97) and 0.92 (0.80–1.00) respectively. In an individual patient data meta-analysis of 115 patients from eight studies, the AUC for the diagnosis of fibrosis stage ≥ 1, ≥ 2, ≥ 3, and 4 were 0.89 (0.81–0.97), 0.90 (0.79–0.93), 0.94 (0.91–0.98) and 0.90 (0.64–0.94) respectively. MRE performed better than TE in a comparative individual patient data meta-analysis of 230 patients [27]. 2D MRE also performs better than serum based indirect biomarkers [28]. Data on diagnostic performance of MRE in selected individual studies are shown in Table 8.1.

Table 8.1 Diagnostic performance of magnetic resonance elastography for the assessment of fibrosis in patients with non-alcoholic fatty liver disease

Study	Study design	Population and prevalence of fibrosis stages	Diagnostic performance
Kim 2013 [29]	Retrospective 2D MRE	142 patients with NAFLD F0 = 50, F1 = 34, F2 = 12, F3 = 10, F4 = 36	AUC = 0.95 for F ≥ 3
Loomba 2014 [24]	Prospective 2D MRE	117 patients with NAFLD F0 = 43, F1 = 39, F2 = 13, F3 = 12, F4 = 10	AUC = 0.84 for F ≥ 1 AUC = 0.86 for F ≥ 2 AUC = 0.92 for F ≥ 3 AUC = 0.89 for F4
Cui 2016 [30]	Prospective 2D MRE	125 patients with NAFLD F0 = 53, F1 = 39, F2 = 12, F3 = 12, F4 = 9	AUC = 0.80 for F ≥ 1 AUC = 0.89 for F ≥ 2 AUC = 0.93 for F ≥ 3 AUC = 0.88 for F4
Imajo 2016 [22]	Prospective 2D MRE	142 patients with NAFLD F0 = 14, F1 = 51, F2 = 32, F3 = 34, F4 = 11	AUC = 0.80 for F ≥ 1 AUC = 0.89 for F ≥ 2 AUC = 0.89 for F ≥ 3 AUC = 0.97 for F4
Loomba 2016 [23]	Prospective 2D MRE (60 Hz)	100 patients with NAFLD F0 = 41, F1 = 32, F2 = 12, F3 = 10, F4 = 5	AUC = 0.85 for F ≥ 1 AUC = 0.88 for F ≥ 2 AUC = 0.92 for F ≥ 3 AUC = 0.98 for F4
	3D MRE (60 Hz)		AUC = 0.86 for F ≥ 1 AUC = 0.84 for F ≥ 2 AUC = 0.93 for F ≥ 3 AUC = 0.98 for F4
	3D MRE (40 Hz)		AUC = 0.85 for F ≥ 1 AUC = 0.86 for F ≥ 2 AUC = 0.98 for F ≥ 3 AUC = 0.99 for F4
Park 2017 [25]	Prospective 2D MRE	104 patients with NAFLD F0 = 47, F1 = 24, F2 = 11, F3 = 13, F4 = 8	AUC = 0.82 for F ≥ 1 AUC = 0.89 for F ≥ 2 AUC = 0.87 for F ≥ 3 AUC = 0.87 for F4
Costa-Silva 2018 [21]	Prospective 2D MRE	49 patients with NAFLD F0 = 21, F1 = 16, F2 = 1, F3 = 8, F4 = 3	AUC = 0.88 for F ≥ 1 AUC = 0.93 for F ≥ 2 AUC = 0.93 for F ≥ 3 AUC = 0.96 for F4

Abbreviations: *2D MRE* 2 dimensional magnetic resonance elastography, *NAFLD* non-alcoholic fatty liver disease, *AUC* area under the curve

Monitoring Treatment Response

MRE has been validated as an exploratory end point in several clinical trials. In an analysis of the data from the phase II trial of selonsertib [31], MRE had an AUC of 0.62 (95% CI: 0.46–0.78) for the prediction of fibrosis improvement, and an

AUC of 0.57 (95% CI of 0.36–0.79 for the prediction of fibrosis progression [32]. In another secondary analysis of the placebo arms of two clinical trials [7, 33], a decrease of ≥5% in body mass index, was associated with a decrease in MRE liver stiffness, while patients who did not lose weight did not show any MRE changes [34].

Predicting Adverse Clinical Outcomes

There are no studies looking at the predictive value of MRE in patients with NAFLD. In a retrospective study of patients with advanced fibrosis (25% had NAFLD), MRE liver stiffness predicted decompensation independently of age, MELD score, serum albumin and hepatitis C diagnosis [35].

Liver*Multiscan*™

Overview

Liver*MultiScan*™ (LMS; Perspectum Diagnostics, Oxford, UK) uses multiple MRI parameters (shMOLLI T1 mapping, T2∗ and PDFF) to provide quantitative measures of liver fibrosis and inflammation, fat and iron. Central to this technology is the correction of the T1 relaxation time, as measured by the shMOLLI technique [36], for iron. T1 is an inherent property of tissues that can change with varying fibrosis and inflammation. T1 is however confounded by the presence of iron. In LMS, the measured T1 is corrected for the amount of iron present (as measured by T2∗), to produce the "iron corrected T1 (cT1)", something that improves the diagnostic accuracy [37]. Even though, this technique has not been validated to the same extent as MRE in patients with NAFLD, it is being used as part of the abdominal imaging protocol in the UK Biobank study [38–41], something that makes it by far the most validated technique in terms of total participants scanned and whose data were subsequently published. Figure 8.1 illustrates this technique in a patient who has undergone bariatric surgery.

The failure rate of LMS is very low (2–5%) [42, 43] in clinical studies. The main reasons for failed scans are participant related factors (e.g. claustrophobia). The failure rate remains at the same low levels when LMS is used in population level studies [38, 39]. LMS cT1 is also a robust technique with excellent reproducibility across scanners and magnet strengths (coefficient of variance 3.3%, bias 6.5 ms, 95% Level of agreement: −76.3 to 89.2 ms) and scan-rescan repeatability (coefficient of variance 1.7%, bias −7.5 ms, 95% Level of agreement: −53.6 to 38.5 ms) [44]. In head to head comparison LMS had superior test re-test repeatability compared to MR elastography and transient elastography [45].

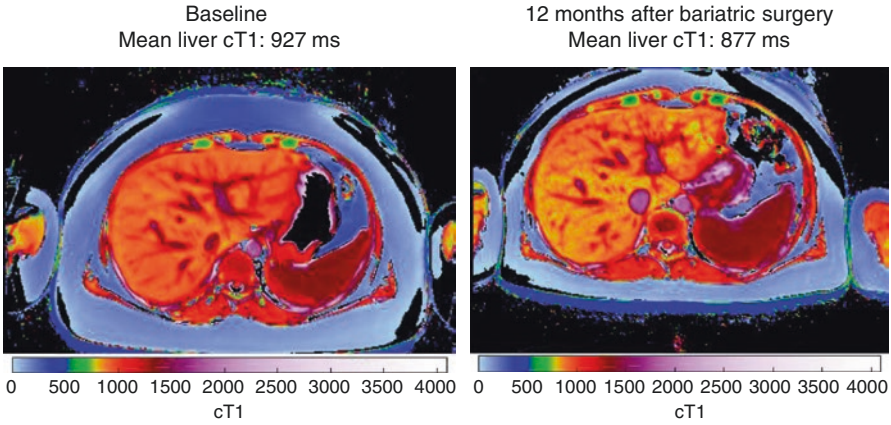

Fig. 8.1 Liver *Multiscan* iron corrected T1 maps. Liver Multiscan produces iron corrected T1 maps that can be used to measure mean cT1. The figure illustrates how the technique can be used to measure change in cT1 after therapeutic intervention, like bariatric surgery

NASH vs. Non-NASH and Staging of NAFLD Fibrosis

Two studies have examined the value of LMS in the staging of fibrosis and the identification of NASH compared to liver biopsy. In a study of 71 patients from one centre [46], LMS cT1 had an excellent diagnostic accuracy for the identification of significant NAFLD as defined by the FLIP consortium algorithm [47] (AUROC 0.89), while there was good performance for the differentiation of NASH vs. simple steatosis (AUROC 0.80). Furthermore, LMS cT1 could identify patients with significant activity (ballooning + lobular inflammation; AUROC 0.83) and cirrhosis (AUROC 0.85). In a two centre study of 50 patients [48], LMS cT1 had moderate diagnostic performance for the separation of NASH vs. simple steatosis (AUROC 0.69), but it must be noted that a different definition of NASH [49] was used in this study. Even though LMS cT1 did not perform as well for the diagnosis of fibrosis compared to alternative tests, it had the highest negative predictive value for the exclusion of significant disease where biopsy could be avoided, and an algorithm in combination with transient elastography had the lowest cost per correct diagnosis [48].

Monitoring Treatment Response

In a study of an engineered fibroblast growth factor 19 analogue (NGM282), both LMS cT1 and PDFF decreased as early as 6 weeks after treatment indicating that this method could be used to assess effectiveness at early time points. This can

improve the design and conduct of clinical trials. LMS cT1 has also been used as a primary end-point in a study without histologic verification of effectiveness, that showed no therapeutic benefit of the investigational product [45]. Along with LMS cT1, there was no improvement in MRE or TE or liver fat measured by LMS PDFF.

Predicting Adverse Clinical Outcomes

LMS has not been specifically tested for the prediction of clinical outcomes in cohorts of patients with NAFLD. In a study including patients with mixed aetiologies (35% NAFLD) and varying degrees of fibrosis, LMS cT1 had a hazard ratio of 9.7 for the prediction of liver related events [50]. In the same study, a model including all three LMS variables (cT1, T2∗ and PDFF) had a hazard ratio of 75.7 demonstrating how the multi-parameter approach in this test can provide improved performance.

It should also be noted that liver T1 was found to correlate with heart failure, atrial fibrillation, and coronary heart disease in the Multi-Ethnic Study of Atherosclerosis [51]. This is important, as it is well documented that cardiovascular disease is the main cause of mortality in patients with NAFLD [2, 3].

Detection of Metabolic Liver Injury (deMILI) MRI

Overview

Detection of metabolic liver injury (deMILI) MRI uses optical analysis of magnetic resonance images to define NASHMRI (0-1) and FibroMRI (0-1), measures of NASH and liver fibrosis respectively. Image acquisition does not require injection of intravenous contrast and include SSFSE-T2 (Single Shot Fast Spin Echo T2-weighted), FAST-STIR (Fast Short inversion Time Inversion Recovery), inPHASE-outPHASE (in and out Phase) and DYNAMIC [52]. Figure 8.2 illustrates the imaging processing and the report for NASHMRI and FibroMRI.

This technique has been validated on 1.5T Phillips and General Electric scanners. Available data suggest that the between scanner reproducibility is good when tested using independent cohorts in Phillips and GE scanners [52]. In small number of patients (n = 9) assessed by both Philips and GE scanners, FibroMRI correctly detected in fibrosis in 3/3 cases and correctly excluded in 5/6 cases using both Philips and GE devices. Furthermore, NASH was correctly diagnosed in 3/4 cases and correctly excluded in 4/5 cases using NASHMRI on data from both scanners [52].

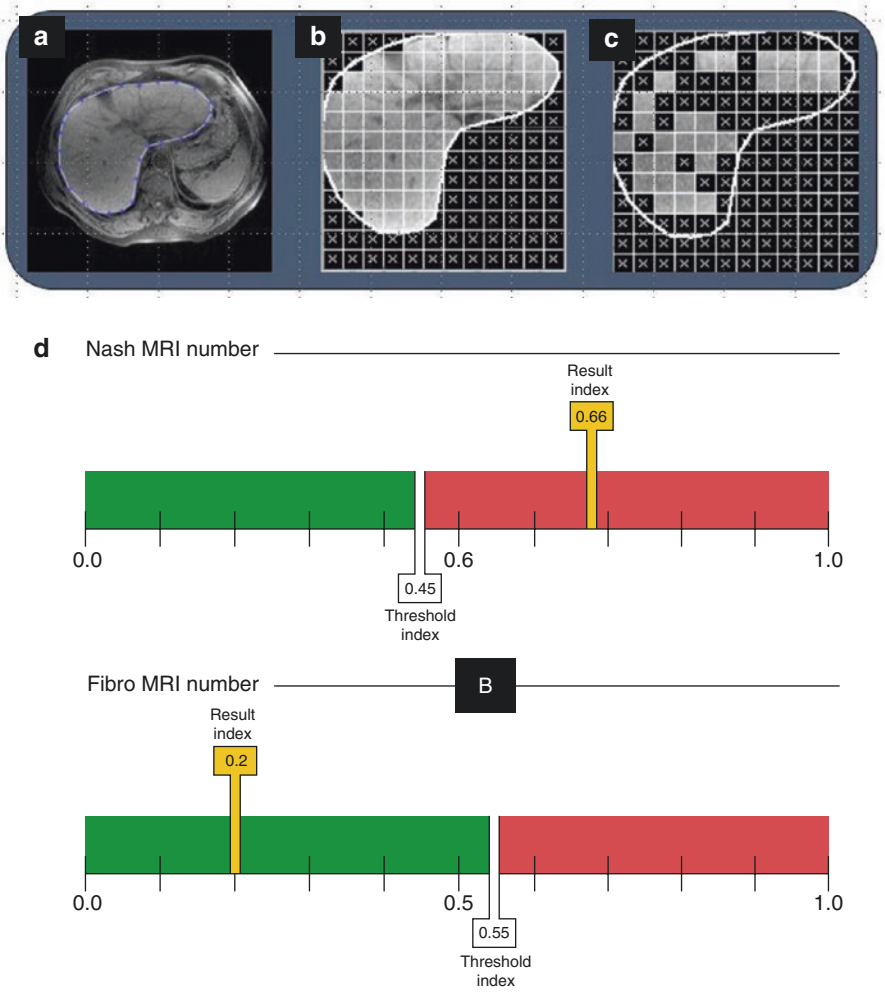

Fig. 8.2 DeMILI image processing and report. The deMILI image processing includes steps for (**a**) the manual outlining of the liver boundary, (**b**) segmentation and overlapping of a grid, (**c**) a process for selection of valid regions of interest. (**d**) The final report is presented as NASHMRI (0-1) where a score above 0.5 indicates NASH and FibroMRI (0-1) where a score above 0.5 indicates significant fibrosis

NASH vs. Non-NASH and Staging of NAFLD Fibrosis

In a prospective study, NASHMRI and FibroMRI were defined based on the most predictive parameters in an estimation and validation cohorts. For the diagnosis of NASH, that was defined histologically based on the overall distribution of lesions especially lobular inflammation and ballooning, NASHMRI had an AUROC of 0.88 (best cut-off 0.5, sensitivity (Se) 0.87, specificity (Sp) 0.74, positive predictive value

(PPV): 0.8, negative predictive value (NPV): 0.82) in the estimation cohort and 0.83 (cut-off 0.5, Se 0.87, Sp 0.6, PPV 0.71, NPV 0.81) in the validation cohort. NASHMRI performed better than Cytokeratin 18 (CK-18) for the diagnosis of NASH [52].

For the diagnosis of significant fibrosis, (F0-F1 vs. F2-F4) FibroMRI had an AUROC of 0.94 (cut-off 0.5, Se 0.81, Sp 0.85, PPV 0.77 and NPV 0.86) in the estimation cohort and 0.85 (cut-off 0.5, Se 0.77, Sp 0.80, PPV 0.67, and NPV 0.87) in the validation cohort. FibroMRI had superior performance compared to serum based fibrosis scores, and similar performance to transient elastography [52].

Dynamic Contrast Enhanced MRI

Overview

Dynamic contrast enhanced MRI relies on the MR signal change in tissues after the injection of intravenous contrast agents. Several contrast agents are available. For the assessment of chronic liver disease, gadoxetic acid is preferred as the liver actively excretes it in bile. In these scans, gadoxetic acid is injected intravenously after acquisition of baseline data. Scans are then acquired at different time points to reflect how the contrast is distributed at the arterial and portal venous phases. Gadoxetic acid is actively taken up by liver cells and then it is selectively excreted into bile. Transmembrane transporters control uptake and excretion. The number of liver cells and their summative level of function ultimately determines how much contrast is taken up into and secreted from the liver. This can be assessed by measuring the resultant change in signal intensity in the liver. Figure 8.3 illustrates how the decrease in signal intensity (T1 in this case) can be used to distinguish normal liver from diseased livers.

This technique requires the injection of intravenous contrast, which is contraindicated in patients with significant renal dysfunction. The advantage of this technique is that it can be applied across scanners and magnet strengths. As it is assessing relative change it requires no further standardisation to make it applicable between scanners. Most of the validation of this technique has been carried out in retrospective studies of patients who were having MR scans as part of their clinical care, so applicability to the wider NAFLD population has not been assessed.

NASH vs. Non-NASH and Staging of NAFLD Fibrosis

There have been some studies showing utility of this technique in animal models of NAFLD/NASH [53–55]. A retrospective human study of 81 patients showed that the relative signal enhancement after contrast injection was associated with lobular inflammation ($p = 0.002$), ballooning ($p = 0.04$) and fibrosis ($p < 0.0001$) but

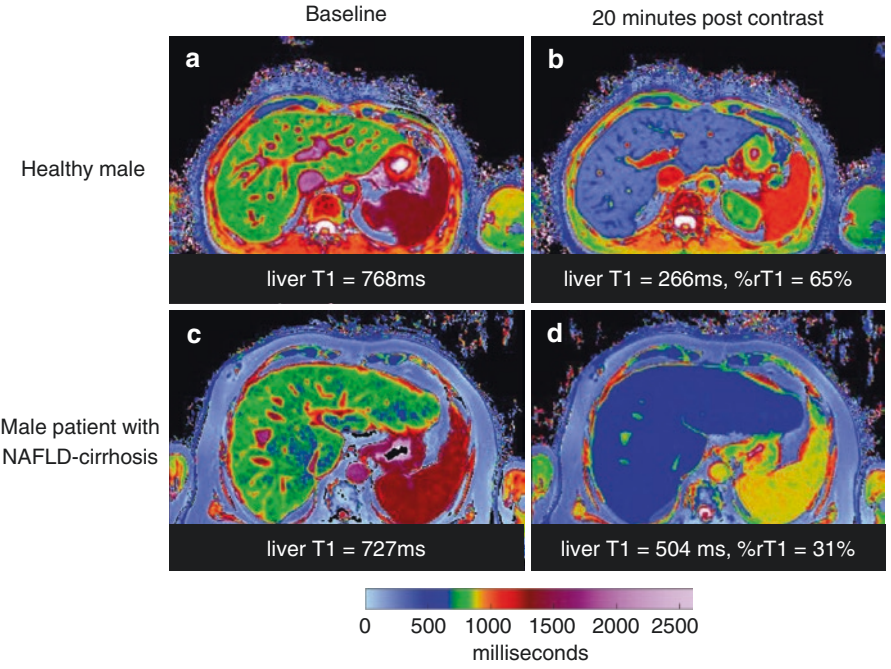

Fig. 8.3 Gadoxetic acid enhanced MRI. The relative reduction in T1 20 min after gadoxetic acid injection in a healthy male and patient with cirrhosis from non-alcoholic fatty liver disease (NAFLD). In the healthy male, T1 decreases from (**a**) baseline of 768 ms to a (**b**) post contrast T1 of 266 ms, a relative reduction (percentagerT1) of 65%, while in the case of the patient, the T1 decreases from (**c**) a baseline of 727 ms to a (**d**) post contrast T1 of 504 ms, a relative reduction of 31%

not with steatosis (p = 0.38) [56]. For the diagnosis of NASH as defined by the Steatosis Activity Fibrosis (SAF) classification [47], this technique had an AUC of 0.85 (threshold 1.24, Se 0.97, Sp 0.63).

Several studies have assessed DCE MRI in mixed cohorts of patients showing some utility in the assessment of liver fibrosis [57], cirrhosis severity [58–60], and liver function [60, 61], including some studies showing superior performance of DCE MRI for the assessment of fibrosis compared to unenhanced T1 and diffusion weighted imaging [62, 63]. However, generalisation of these results to NAFLD patients must not be assumed.

A related approach to using gadolinium based contrast agents is to use iron containing contrast agents. Superparamagnetic iron oxide particles have been tested, but these have since been taken off the market [64]. More recently, there has been some interest in ultrasmall superparamagnetic iron oxide particles. The iron containing contrast leads to changes in tissue R2∗ which can be measured. In a small, prospective, proof-of-concept study, the AUC for the diagnosis of NASH vs. simple steatosis was 0.87 (95% CI 0.72–1.0) [65]. However, the post contrast scans are acquired 72 h after injection something that is impractical in clinical practice.

Diffusion Weighted Imaging

Overview

Diffusion weighted imaging (DWI) uses MRI acquisition and analysis techniques to track diffusion of water in tissues. Quantitative measures of diffusion can be produced by measuring the magnitude (apparent diffusion coefficient; ADC) and directionality (fractional anisotropy) of diffusion. The accumulation of steatosis, inflammation and fibrosis can lead to changes in water diffusion and these can be measured using various DWI techniques. Intravoxel incoherent motion (IVIM) is a DWI method that can account for the diffusion signal contributed from blood flowing in vascular beds [66].

The failure rate of this technique was up to 17.5% in one study [67]. The method of analysis can also have a significant impact on results [68].

NASH vs. Non-NASH and Staging of NAFLD Fibrosis

A study of 59 patients with type 2 diabetes mellitus and NAFLD evaluated the IVIM parameters of "pure molecular diffusion; D", "perfusion related diffusion, D∗ and "perfusion fraction; f". The study found only moderate diagnostic accuracy for the diagnosis of NASH (AUC 0.74 for D, 0.68 for D∗, 0.61 for f) and fibrosis (AUC 0.69 for D, 0.68 for D∗, 0.62 for f) [67]. In a separate study of 89 patients with NAFLD, steatosis and fibrosis had significant and independent effects on D and f [68]. The effects of steatosis have also been observed in other studies [69–72].

In an interesting retrospective study of 15 patients (only 2 with NAFLD), a method is proposed by which IVIM can be used to generate a "virtual elastogram" based on a calibrated relationship between ADC and liver elasticity [73]. This lacks prospective validation in patients with NAFLD but could provide an added advantage to MRE as it could potentially produce equivalent data without the need for additional hardware.

Conclusions

The field of MR based biomarkers is relatively new compared to serum-based biomarkers and ultrasound based elastography techniques. Of the techniques that have been reviewed in this chapter, MRE (+PDFF for fat) and LMS have had most validation in NAFLD and they show promise for further clinical utility. MRE has the best performance for assessment of late stages of fibrosis. PDFF for liver fat content quantification is emerging as an important parameter for predicting histological response.

How various MR techniques are utilised in clinical pathways and clinical trials remains to be determined. Current recommendations [17] favour application of MR based techniques as a third tier of non-invasive tests after serum based and ultrasound elastography. While this approach may be more practical there are no cost effectiveness data to support it and it could be that application of MR based techniques "up-front" are more cost effective if they have superior diagnostic accuracy.

One other area that needs further attention is the validation of pre-defined thresholds to be used in different situations (contexts of use). For example, there is growing evidence that a relative reduction of 30% in liver fat content predicts histological response but data are still lacking on prospective validation of predefined cut-offs for varying fibrosis severities. Data on the prognostic value of MR based biomarkers in NAFLD cohorts are also needed.

MR based biomarkers will certainly have a role in the assessment of patients with NAFLD as the data reviewed here demonstrate advantages in some key areas beyond diagnostic accuracy. MR based biomarkers are robust with excellent reproducibility and repeatability, can be applied at population level as in the case of Liver *Multiscan* being used in the UK Biobank imaging study. Further technical improvements are also possible as in the use of diffusion weighted imaging to perform "virtual elastography".

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Chapter 9
Extracellular Vesicles in Non-alcoholic Fatty Liver Disease: Key Players in Disease Pathogenesis and Promising Biomarker Tools

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Abbreviations

2D-SWE	2-dimensional shear wave elastography
ADSCs	Adipose-derived stem cells
AFM	Atomic force microscopy
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AUC	Area under the receiver operating curve
cryo-EM	Cryo-electron microscopy
CAP	Controlled attenuation parameter
CCN2	Connective tissue growth factor 2
CDAA	Choline-deficient L-amino acid defined
CHC	Chronic hepatitis C
CK18	Cytokeratin 18
CXCL10	C-X-C motif ligand 10
DLS	Dynamic light scattering
ELISA	Enzyme-linked immunosorbent assay
EVs	Extracellular vesicles
FFAs	Free fatty acids
FGF21	Fibroblast growth factor 21
GPI	Glycosylphosphatidylinositol
hrFC	High-resolution flow cytometer
HCC	Hepatocellular carcinoma
HFS	Hepamet fibrosis score
HMGB1	High mobility group box 1
HSCs	Hepatic stellate cells
iNKT	Invariant natural killer T cells
Ihh	Indian hedgehog
ILVs	Intraluminal vesicles
ISEV	International society for extracellular vesicles
LFS	Liver fibrosis score
miRNAs	microRNAs
mtDNA	Mitochondrial DNA
MLK3	Mixed lineage kinase 3
MRE	Magnetic resonance elastography
MRI-PDFF	Magnetic resonance imaging-derived proton density fat fraction
MVBs	Multivesicular bodies
MVEs	Multivesicular endosomes
MVs	Microvesicles
ncRNAs	Non-coding RNAs
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis

NFS	NAFLD fibrosis score
NTA	Nanoparticle tracking analysis
pSWE	Point shear wave elastography
PA	Palmitic acid
PPARs	Peroxisome proliferator-associated receptors
ROCK1	Rho-associated, coiled-coil-containing protein kinase 1
S1P	Spingosine-1-phosphate
SAF	Steatosis activity fibrosis
SEM	Scanning electron microscopy
Shh	Sonic hedgehog
TE	Transient elastography
TEM	Transmission electron microscopy
TGFβ	Transforming growth factor β
TLR9	Toll-like receptor 9
TRAIL	TNF-related apoptosis inducing ligand
TRPS	Tunable resistive pulse sensing
VCAM1	Vascular cell adhesion molecule 1

Introduction

Non-alcoholic fatty liver disease (NAFLD) represents a common condition characterized by the pathologic accumulation of fat within more than 5% of hepatocytes, in the absence of other forms of liver disease, such as viral infections or excessive alcohol intake, among others [1]. NAFLD encompasses a spectrum of liver lesions ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). Hepatic or simple steatosis is usually benign and considered one of the earliest and less severe stages of NAFLD, exhibiting a relatively favorable clinical course. In about 5–10% of patients, hepatic steatosis may progress to NASH, a more malignant state that is prone to further progress to cirrhosis and hepatocellular carcinoma (HCC), substantially contributing to liver-related morbidity and mortality [1].

NAFLD is currently the most prevalent liver disease worldwide, affecting around one-fourth of the general population [2]. In fact, several NAFLD epidemiological studies have been conducted in both the United States and Europe, indicating that over than 64 million people in the United States are believed to possess NAFLD [3–5]. In Europe, the prevalence of NAFLD varies between countries. In Spain, a 2010 multicenter cross-sectional population study revealed that the overall prevalence of NAFLD was 25.8% [6]. In Romania, the estimated prevalence was 20% [7] while a Greek study stated that 31.3 and 39.8% of liver biopsies from 498 individuals showed hepatic steatosis or NASH, respectively [8]. In this regard, it was predicted that over 52 million individuals would be affected by NAFLD in Germany, France, Italy and the United Kingdom [3]. Although NAFLD incidence is increasing worldwide, it is most noticeable in specific population groups, particularly those

with metabolic comorbidities. NAFLD prevalence is increased in patients with type 2 diabetes, who also display an increased risk for development of NASH [9, 10]. Two major European studies reported a NAFLD prevalence rate between 42.6 and 69.5% in patients with type 2 diabetes [11, 12]. Obese individuals are also more likely to develop NAFLD. In particular, 65.7% of obese patients undergoing bariatric surgery have NAFLD, with 33.6% also evidencing NASH [13]. Overall, patients with metabolic syndrome, pathological conditions characterized by abdominal obesity, hypertension, dyslipidemia and glucose intolerance are at increased risk of developing NAFLD [14].

NAFLD is thought to increase cardiovascular risk, as most NAFLD patients die from cardiovascular-related problems [15–19]. Indeed, the number of NAFLD patients with advanced disease (i.e., cirrhosis, end-stage liver disease, HCC), a major cause of liver disease-related morbidity and mortality, is alarmingly increasing [20, 21]. Both liver-specific and overall mortality among NAFLD patients is estimated at 0.77 and 11.77 per 1000 person-year, respectively, further increasing in patients with NASH (15.44 and 25.56 per 1000 person-year, respectively) [2]. Of note, NASH is currently the second indication for liver transplantation among all chronic liver diseases and is expected to become the leading indication for transplantation in the next decades [22, 23]. In parallel with NAFLD pathogenesis remaining incompletely understood, one of the major clinical challenges is the difficulty of obtaining differential diagnosis between the different disease severity stages, and also the identification of patients that might be at higher risk for disease progression. As such, there is a substantial unmet need for novel non-invasive and accurate tools that might allow the diagnosis and risk stratification of NAFLD patients. In this regard, extracellular vesicles (EVs) are emerging as promising molecular targets and biomarkers, being involved in disease pathogenesis and harboring diagnostic and prognostic potential. In this chapter, we will review the most recent findings concerning the role of EVs in NAFLD pathogenesis and in diagnosis/prognosis.

NASH Diagnosis and Monitoring: Current Approaches

Liver biopsy is still the gold standard procedure to undoubtedly identify and stage NAFLD. Kleiner and Blunt proposed a scoring system, named the NAFLD Activity Score (NAS), which is calculated by the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3) and hepatocyte ballooning (0–2) [24]. Consequently, NAS ranges from 0 to 8 and a total score of 0–2 is considered a non-NASH diagnosis while scores greater or equal to 5 are diagnosed as NASH. Of note, fibrosis was not included as a component of the activity score due to its irreversible nature and because it was thought to only result from disease progression. Still, fibrosis is often present in NASH; to that matter, the Steatosis Activity Fibrosis (SAF) score, which does not sub-classifies NAFLD based solely in NASH, was proposed. This score evaluates three variables: steatosis, on a scale of 0–3 (S0: <5%, S1: 5–33%, S2: 34–66%, S3: >67%); ballooning and lobular inflammation, each graded between 0 and 2 and then summed, representing activity (A0–A4), and fibro-

sis, scored between 0 and 4 (F0: none, F1: perisinusoidal or periportal, F2: perisinusoidal and periportal, F3: bridging or F4: cirrhosis) [25]. This new score was shown to decrease intra-observer variation among pathologists and includes the fibrosis component in the final decision which, although not required for the diagnosis of NASH, is now considered to represent the best predictor of advanced liver disease and mortality [26]. Nonetheless, the SAF score still requires a liver biopsy to be performed, carrying several intrinsic limitations, including invasiveness, poor acceptability, variability and cost [27]. Therefore, alternative non-invasive strategies have been proposed in order to achieve a more accurate diagnosis and to assist in the management of NAFLD patients [27, 28]. For instance, the Hepamet Fibrosis Score (HFS), mainly based on serum markers, was recently developed an validated in a cohort of 2453 patients with NAFLD, presenting area under the receiver operating characteristic (AUC) values greater than other fibrosis score systems (the NAFLD fibrosis score [NFS] and FIB-4) [29]. Currently, 2 different complementary approaches are being used as non-invasive methods in the diagnosis and management of NAFLD: a "biological" approach, which mainly relies on the quantification of serum biomarkers, and a "physical" approach, related with the measurement of the intrinsic physical properties of the liver parenchyma (liver stiffness) by different imaging techniques [27].

Although the vast majority of NAFLD patients are clinically asymptomatic, approximately 20% display elevated liver enzymes [30]. Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) may present normal to moderate elevations—about 1.5–2 times the upper limit—although these are considered poor markers of fatty liver disease [31, 32]. Several predictive models (SteatoTest [33], Fatty Liver Index [34], Hepatic Steatosis Index [35], lipid accumulation product [36], the Index of NASH [37] or the NAFLD Liver Fat Score [38], based on the differential combination of serum transaminases, triglyceride levels and clinical information have been developed (reviewed in [39]) to help in the diagnosis of steatosis. It should be noted that diagnostic performances of each test are difficult to compare since they are based against different standards, namely liver biopsy or imaging techniques. Still, in a cohort of 324 patients with suspected NAFLD and liver biopsy, the Fatty Liver Index, NAFLD Fat Score and Hepatic Steatosis Index were retrospectively evaluated, providing similar AUC values (0.83, 0.80 and 0.81, respectively) [40]. However, these scores are still not widely used as they fail to provide substantial additional information when compared with current clinical, laboratory and imaging studies, and await further validation studies. Cytokeratin-18 (CK18) fragments have been extensively studied as biomarkers for NASH diagnosis. CK18 fragments are released from apoptotic hepatocytes and the caspase-cleaved fragment M30 is detected by enzyme-linked immunosorbent assay (ELISA) in the serum of patients with NASH, with a AUC value of 0.83 [41]. In two subsequent meta-analyses, the pooled AUC for CK18 to predict NASH was 0.82 (median sensitivity and specificity of 66–78% and 82–87%, respectively) [42, 43]. Nonetheless, there are still several drawbacks with regards to using CK18 as a diagnosis biomarker for NASH, restraining its translation into clinical practice. In particular, there is significant variability in the suggested cutoffs and diagnostic accuracy among studies [44], no commercially available clinical tests [45] and a rather limited sensitivity when used alone [46]. On

this note, the diagnostic accuracy of CK18 is increased when used in combination with sFas levels [47], uric acid [48], adiponectin and resistin [49, 50], ALT or the presence of metabolic syndrome [51], among others. Some models have been proposed but most of them were studied in small and highly selected populations (morbidly obese patients) and, as such, need to be further validated. Serum metabolomics was also shown to be of great value in the diagnosis of NAFLD/NASH and recent studies have reported specific serum metabolomic signatures that allowed the specific diagnosis of NAFLD (OWL Liver Care) and the differential diagnosis of steatosis and NASH (OWL Liver Test) [52, 53] as well as between NASH subtypes [54], that are currently commercialized by OWL Metabolomics.

Imaging techniques, either ultrasound- or magnetic resonance-based, are widely used in NAFLD diagnosis. Conventional ultrasonography is currently the most commonly used imaging technique for the diagnosis of steatosis due to its general availability [27]. With ordinal ultrasonography scores, steatosis may be subjectively categorized as mild, moderate and severe [55, 56] Importantly, in a large meta-analysis, including 34 studies and 2815 patients with suspected/diagnosed liver diseases, pooled sensitivities and specificities of ultrasonography to differentiate moderate/severe fatty liver and absence of steatosis, were 85% and 93%, respectively [57]. Still, in daily practice, ultrasonography is only used to provide a diagnosis of presence or absence of steatosis, only being capable of detecting liver fat amounts greater than 2.5–20% [58] and displaying lower accuracy in obese patients with concomitant renal disease [59, 60]. According to the European Guidelines for the management of NAFLD [20], ultrasonography constitutes the first choice imaging technique to monitor steatosis in adults at risk for disease development. Controlled Attenuation Parameter (CAP) is a new non-invasive transient elastography (TE)-based imaging technique for the staging of steatosis. Good inter-observer reproducibility was reported (concordance rates between observers of 0.82–0.84) [55, 56], and in a cohort of patients with chronic liver diseases (15% with NAFLD), steatosis was accurately detected by CAP. Still, this technique was not precise enough to discern between the different stages of steatosis [61]. Furthermore, CAP determinations might be influenced by the presence of covariates, such as the body mass index or diabetes [27]. Finally, magnetic resonance-based imaging techniques have been reported as the most accurate available imaging techniques to quantify liver fat and fibrosis. Magnetic resonance imaging proton density fat fraction (MRI-PDFF) is highly accurate (AUC: 0.950) [62], and reproducible, fast, and allows for evaluation of the entire liver, quantification of fat content, and stratification of steatosis (with excellent sensitivity for detecting mild steatosis) [63]. This technique has been validated in several studies [64, 65] and is emerging as the gold standard for the quantification of liver fat. However, it is not widely available, it is rather expensive and measurements are presumed to be affected by food intake [66]. Novel methodologies, including point shear wave elastography (pSWE) and 2-dimensional shear wave elastography (2D-SWE) are now being evaluated [67–69].

Of note, the differential diagnosis of simple steatosis and NASH by imaging techniques remains challenging, as MRI-PDFF cannot effectively detect liver inflammation and ballooning, nor NASH resolution or fibrosis improvements [70]. The same holds true for magnetic resonance elastography (MRE) [71–75] and TE [72, 76–78].

Overall, there is still no biomarker or imaging method capable of accurately diagnosing, staging and performing the follow-up of NAFLD (including fibrosis) for which new approaches are eagerly awaited. In this regard, EVs are emerging as novel potential NAFLD biomarkers, while also participating in disease pathogenesis.

Extracellular Vesicles

EVs are a heterogeneous population of membrane vesicles ranging from 30 nm to 2 μM in diameter, secreted by diverse cell types and containing distinct biomolecules, including proteins, nucleic acids and lipids [79–82]. The first reports on EVs considered them membrane debris with no biological significance, and a way to eliminate needless compounds by the cells [83]. However, new evidence demonstrating their potential to stimulate adaptative immune responses [84, 85], opened their role in intercellular communication. In the last decade, this is an emerging field which is exponentially increasing, with special interest in their capacity to exchange components between cells and acting as signaling vehicles (Fig. 9.1).

Based on the current knowledge of their biogenesis, EVs are classified as exosomes, microvesicles (MV) and apoptotic bodies (Fig. 9.2) [79–82, 85, 86]. **Exosomes** (30–200 nm) are formed as intraluminal vesicles (ILV) by the inward

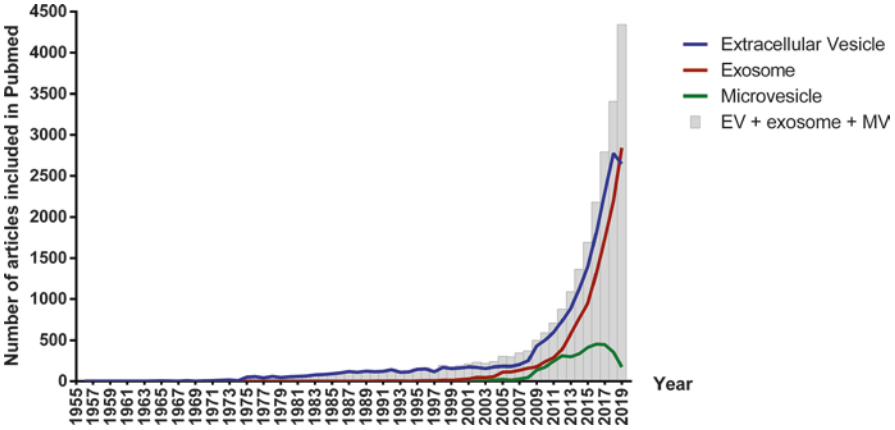

Fig. 9.1 Timeline (1955–2019) of articles referring to extracellular vesicles, microvesicles and exosomes in PubMed. (https://www.ncbi.nlm.nih.gov/pubmed/). Accession date: December 2019
Abbreviations: *EV* extracellular vesicle; *MV* microvesicle

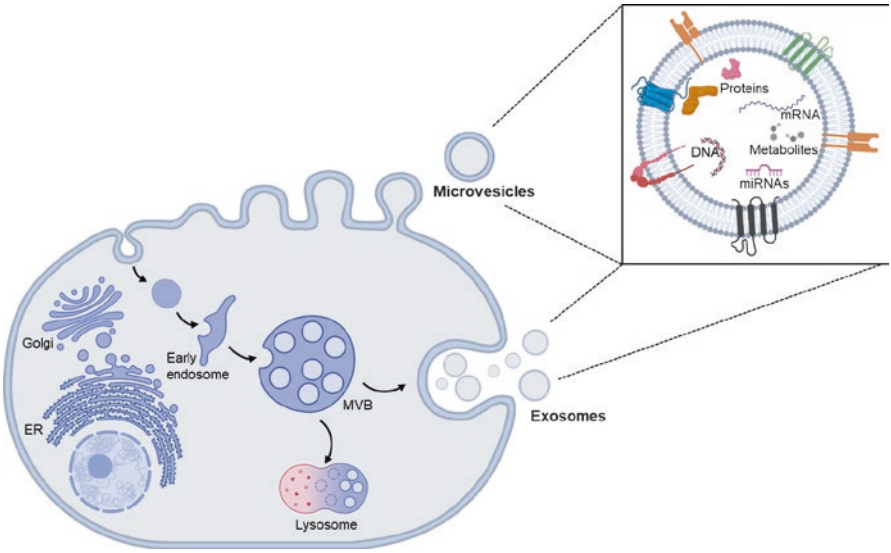

Fig. 9.2 Biogenesis, overall composition and release of EVs. MVs are produced by the outward budding and fission of the plasma membrane, whereas exosomes are formed by the inward budding of the multivesicular body and released upon fusion of multi-vesicular bodies with the plasma membrane. EVs are lipid-bilayer membrane vesicles which contain cytoplasmic proteins, lipid raft-interacting proteins, membrane proteins, lipids, metabolites, DNA and different types of RNA. Abbreviations: *ER* endoplasmic reticulum; *MVB* multivesicular body

budding of the endosomal membrane during maturation of multivesicular endosomes (MVE). They are secreted from the lumen of late endosomes, also called multivesicular bodies (MVBs), to the extracellular space by fusion of MVBs with the plasma membrane. **Microvesicles** (50–1000 nm) are generated by the outward budding and fission of the plasma membrane and the subsequent release of vesicles into the extracellular space. Finally, **apoptotic bodies** (800–5000 nm) are released by cells when plasma membrane blebbing occurs during programmed cell death. All EVs may contain cytoplasmic proteins, lipid raft-interacting proteins, membrane proteins, lipids, metabolites, DNA and different types of RNA, including mRNAs, microRNAs (miRNAs) and other non-coding RNAs (ncRNAs) [85, 87]. Their molecular cargo varies widely among cell types and conditions (e.g., physiology/pathology), directly affecting the fate and function of these membrane vesicles [80]. EVs are found in all biological fluids, including serum, plasma, urine, saliva, and bile, among others, as well as in culture supernatants [81, 82]. However, despite their different biogenesis, once they reach the extracellular zone, exosomes and microvesicles display a similar appearance, overlapping in size, and often presenting a common biomolecular composition [80]. Therefore, it is difficult to ascertain the origin of EVs when they are isolated from the extracellular medium or from diverse biological fluids.

A wide variety of methods have been proposed for the isolation of EVs from extracellular fluids: differential centrifugation/ultracentrifugation, flotation on density gradients, separation by size exclusion chromatography, precipitation with different polymers, filtration and antibody-based purification (immuno-affinity) [79, 88]. These methods allow the separation of EVs from non-vesicular entities, such as protein aggregates, lipoparticles, viruses and cell debris, with different rates of success [80]. Given the heterogeneous EV population, each purification method will result in enrichment of specific EV subpopulations, with distinct recovery/specificity rates [88]. Nonetheless, combination of multiple isolation procedures is capable of specifically separating subpopulations of vesicles based on their size, density, surface proteins, sugar, lipid composition or other biophysical properties, such as surface charge. Even so, considering that a single optimal separation method or a gold standard is not yet defined, the isolation method should be chosen based on the downstream application and on the scientific question that is being addressed [79, 88]. Once EVs are obtained, they should be properly characterized using multiple, complementary techniques, in order to obtain reproducible results. In this regard, the International Society for Extracellular Vesicles (ISEV) regularly publishes minimal requirement guidelines for the study of EVs, focusing on adequate and standardized characterization [79, 88]. In parallel, a consortium called EV-TRACK (transparent reporting and centralizing knowledge in extracellular vesicle research) has been gathered to build a crowdsourcing knowledgebase (http://evtrack.org/) that centralizes EV biology and methodology with the goal of stimulating authors, reviewers, editors and funders to put experimental guidelines into practice [89].

The characterization of EVs should include determination of its morphology, size and concentration, as well as reporting of the components typically associated with EVs, particularly membrane proteins [79, 88]. Scanning electron microscopy (SEM), transmission electron microscopy (TEM), cryo-electon microscopy (cryo-EM) and atomic force microscopy (AFM) images enable the analysis of EV morphology and size, while also providing information on the heterogeneity of the EV preparation. Particle number and size can be measured quantitatively by analyzing large numbers of single EVs with light scattering technologies, such as nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS), as well as with high resolution flow cytometry (hrFC) or tunable resistive pulse sensing (TRPS). The analysis of EV-associated proteins is typically performed through immunoblotting, flow cytometry and/or mass spectrometry. It has been described that transmembrane or glycosylphosphatidylinositol (GPI)-anchored proteins localized in plasma membrane and/or endosomes, such as tetraspanins (CD63, CD81, others) or integrins, may be considered as markers of any type of EVs, as their presence demonstrates the existence of the lipid-bilayer structure characteristic of EVs. Additionally, cytosolic proteins with membrane-binding ability, such as ESCRT-I/II/III (e.g., TSG101), heat shock proteins, ALIX and ARF6 are also commonly found in EVs, given the nature of its biogenesis. Besides proteins, phospholipids found in lipid bilayers are

also potential positive controls for identifying EVs. Still, these might be non-specific, as other particles such as lipoproteins can also contain phospholipids. It remains important to clarify the ratios of cholesterol, sphingomyelin, ceramide, and phosphatidylcholine/ethanolamine/inositol found in EVs, and how these differ from the ratios found in lipoproteins. In fact, there are still no markers capable of accurately distinguishing between every different EV subtype [80, 85, 88].

The mechanisms involved in EV-mediated cell-to-cell communication are vast and still incompletely understood. When interacting with target cells, EVs may modulate cellular signaling pathways in a pleiotropic manner, including the direct activation of cell surface receptors *via* protein and bioactive lipid ligands, or by merging their membrane contents into the recipient cell [80]. This type of communication is believed to occur in both physiological conditions as well as in pathological states [85]. In physiological conditions, EVs participate in the maintenance of stemness [90], tissue repair [91], blood coagulation [92], immune surveillance [93], neuronal plasticity [94] and several other physiological functions [85]. In turn, EVs may contribute to tumorigenesis by inducing abnormal cell proliferation [95], stimulating tumor growth [96], promoting extracellular matrix remodeling [97], and facilitating tumor metastasis [98] and immune escape [93]. Beyond cancer, EVs appear to also play a role in the spread of different pathogens, in the local propagation of neurodegenerative diseases, and in several liver diseases, including NAFLD (reviewed in [99, 100]).

Role of Extracellular Vesicles in NAFLD Pathogenesis

As central mediators of cell-to-cell communication, EVs have recently arisen as novel players in NAFLD pathogenesis and progression (Fig. 9.3; Table 9.1). Data from different diet-induced animal models of NASH have shown that EV concentration increases with disease progression, in a time-dependent manner [101–103]. This may result from accumulation of lipotoxic lipids and their downstream mediators in the liver, already shown to increase the capacity of hepatocytes to form and release different types of EVs [102–105]. EVs can then be internalized by macrophages, neutrophils and monocytes, leading to their activation and recruitment to the liver, promoting and exacerbating the inflammatory responses observed in NASH. In fact, palmitic acid (PA) and lysophosphatidylcholine were shown to increase the release of microvesicles carrying TNF-related apoptosis inducing ligand (TRAIL) ligand from both mouse and human hepatocytes [105]. In mice, this promotes the expression of pro-inflammatory cytokines IL-1β and IL-6 in bone marrow-derived macrophages, in a rho-associated, coiled-coil-containing protein kinase 1 (ROCK1)-dependent manner [105]. Of note, administration of fasudil, a ROCK1 inhibitor, to mice with NASH decreases the amount of EVs in the serum as well as liver injury, inflammation and fibrosis. PA-stimulated hepatocytes have also been shown to release EVs enriched in ceramide, which increase macrophage recruitment to the liver *via* sphingosine-1-phosphate (S1P). Indeed, increased levels

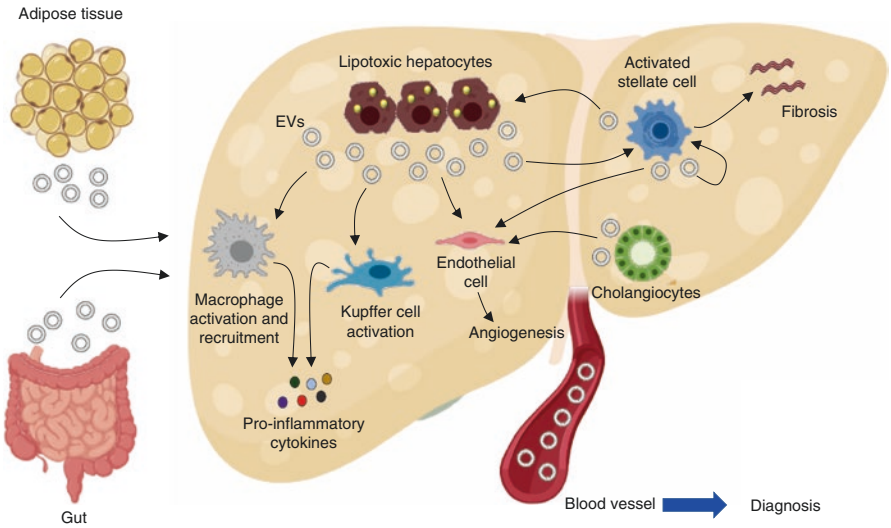

Fig. 9.3 Role of EVs in NAFLD pathogenesis. EVs are released by lipotoxic hepatocytes, thus contributing to the recruitment and activation of macrophages, Kupffer and stellate cell activation, as well as angiogenesis, through targeting of endothelial cells. Hepatic stellate cells and cholangiocytes might also secrete EVs, thus also contributing for disease progression. In addition, adipose tissue- and gut-derived EVs are also known to target the liver and contribute to NAFLD. Finally, increased blood concentration of EVs might help in NAFLD diagnosis. Abbreviations: *EVs* extracellular vesicles

Table 9.1 Extracellular vesicles (EVs) involved in cell-to-cell and organ crosstalk during NAFLD pathogenesis

EV type	Source cells	Target cells	EV cargo	Modulated targets in recipient cells	Models	Ref.
EVs	Liver (hepatocytes)	Bone marrow-derived macrophages	Ceramide	S1P	*In vitro*; *In vivo* (mouse); patients	[102]
Microvesicles	Liver (hepatocytes)	Liver (endothelial cells)	Vanin-1	–	*In vitro*; *In vivo* (mice)	[103]
EVs (exosomes)	Liver (hepatocytes)	Bone marrow-derived macrophages	CXCL10	–	*In vitro*; *In vivo* (mice)	[104]
Microvesicles	Liver (hepatocytes)	Bone marrow-derived macrophages	TRAIL	IL-1β and IL-6	*In vitro*; *In vivo* (mouse); patients	[105]
EVs	Liver (hepatocytes)	Neutrophils and Kuppfer cells	mtDNA	TLR9	*In vivo* (mice); patients	[107]

(continued)

Table 9.1 (continued)

EV type	Source cells	Target cells	EV cargo	Modulated targets in recipient cells	Models	Ref.
Exosomes	Liver (hepatocytes)	Liver (HSCs)	miR-192	Fibrogenic markers (α-SMA, TGF-β, and Col1α1)	*In vitro*	[110]
Exosomes	Liver (hepatocytes)	Liver (HSCs)	–	Fibrogenic markers (Col1α1, Col3α1, MMP-2, and TIMP-1)	*In vitro*; *In vivo* (mice)	[111]
EVs	Liver (hepatocytes)	Liver (HSCs)	miR-128-3p	PPAR-γ	*In vitro*; *In vivo* (mice)	[112]
Exosomes	Liver (HSCs)	Liver (hepatocytes and HSCs)	miR-214	CCN2	*In vitro*	[113]
Exosomes	Liver (HSCs)	Liver (HSCs)	CCN2	–	*In vitro*	[114]
Exosomes	Liver (HSCs and hepatocytes)	Liver (HSCs)	Twist1	miR-214 (CCN2)	*In vitro*	[115]
Exosomes	VAT (adipocytes)	Liver (hepatocytes and HSCs)	–	TGF-β pathway genes (TIMP-1, TIMP-4, integrin αvβ-5, integrin αvβ-8, PAI-1, Smad-3, MMP-7 and MMP-9)	*In vitro*; patients	[117]
Exosomes	BAT (adipocytes)	Liver	miR-99b	FGF-21	*In vivo* (mouse)	[118]
Exosomes	ADSC (adipose-derived stem cells)	Macrophages	STAT3	Arginase-1	*In vivo* (mouse)	[119]
Exosomes	Intestine	Liver	HMGB1	–	*In vivo* (mouse)	[121]
Exosomes	Liver (MF-HSC and cholangiocytes)	Liver (endothelial cells)	Shh and Ihh	–	*In vitro*; *In vivo* (mice)	[122]

EV extracellular vesicles; *VAT* visceral adipose tissue; *HSC* hepatic stellate cells; *TGF-β* transforming growth factor-β; *TIMP-1* tissue inhibitor of matrix metalloproteinase-1; *TIMP-4* tissue inhibitor of matrix metalloproteinase-4; *PAI-1* plasminogen activator inhibitor-1; *MMP-7* matrix metalloproteinase-7; *MMP-9* matrix metalloproteinase-9; *BAT* brown adipose tissue; *FGF21* fibroblast growth factor-21; *STAT3* signal transducer and activator of transcription 3; *HMCB1* high mobility group box-1; *TRAIL* tumor necrosis factor-related apoptosis-inducing ligand; *Il-1β* interleukin 1 beta; *Il-6* interleukin 6; *SIP* sphingosine-1-phosphate; *CXCL10* C-X-C motif ligand 10; *mtDNA* mitochondrial DNA; *TLR9* toll-like receptor 9; *α-SMA* α-smooth muscle actin; *Col1α1* Collagen, type I, alpha 1; *Col3α1* Collagen, type III, alpha 1; *MMP-2* matrix metalloproteinase-2; *PPAR-γ* peroxisome proliferator-activated receptor-γ; *CCN2* pro-fibrogenic connective tissue growth factor; *MF-HSC* myofibroblastic hepatic stellate cells; *Shh* Sonic hedgehog; *Ihh* Indian hedgehog

of ceramide- and S1P-enriched EVs were reported in plasma from both mice and patients with NASH [102]. Importantly, in mice fed a high-fructose, -saturated fat and -cholesterol diet, blocking of S1P was shown to improve liver histology, namely reducing hepatocyte ballooning and inflammatory foci, in parallel with reduced hepatomegaly, serum transaminases and accumulation of hepatic macrophages [106]. Finally, EVs carrying C-X-C motif ligand 10 (CXCL10), a potent chemokine that is released by lipotoxic hepatocytes in a Mixed Lineage Kinase 3 (MLK3)-dependent manner, have also been shown to associate with macrophage recruitment [104]. In addition, the majority of mitochondrial DNA (mtDNA) released from hepatocytes circulate within microparticles and promote activation of neutrophils and kupffer cells through toll-like receptor 9 (TLR9) [107]. More recently, it was shown that the NLRP3 inflammasome is activated by microvesicles released from fat-laden cells undergoing lipotoxicity, either in hepatocytes and macrophages, further reinforcing the role of EVs in disease progression from simple steatosis to NASH [108]. On that note, the transplant of circulating EVs from high fat-fed mice to chow-fed mice was shown to induce accumulation and consequent activation of myeloid cells in the liver, thus promoting liver inflammation and injury [109].

Injured hepatocytes are able to communicate with other liver cell types, such as hepatic stellate cells (HSCs), thus triggering the expression of fibrosis-related genes [110, 111]. In fact, EVs released from PA-stimulated hepatocytes have been shown to enhance the expression of fibrosis markers in HSCs. Among its cargo, these EVs were shown to carry several miRNAs, including miR-192, already associated with NAFLD progression and liver fibrosis [110]. Interestingly, incubation of HSC cultures with plasma EVs from mice fed a high-fat diet, triggered their activation, again underscoring EVs as key cell-to-cell communication mediators. In addition, in a dietary murine model of NASH, mice treated with thiazolidinediones, a group of insulin sensitizers that activate peroxisome proliferator-activated receptors (PPARs), exhibited reduced secretion of EVs from hepatocytes, thereby impairing HSCs activation [111]. This emphasizes the crucial role of PPARs inhibition, especially PPAR-γ, in the phenotypical switch of HSCs from quiescent to their active form. In agreement, hepatocyte-derived EVs released during lipotoxicity are enriched with miR-128-3p, a miRNA that direct targets PPAR-γ and promote HSCs migration, proliferation and activation, therefore contributing to fibrosis and NAFLD progression [112].

Hepatocytes are not the only source of EVs in the liver that actively contribute to this type of paracrine communication. In fact, both mouse and human HSCs release EVs that target hepatocytes and HSCs themselves [113–115]. For instance, it was shown that under fibrotic conditions, HSCs release EVs carrying lower levels of miR-214, when compared with physiological situations, which directly target connective tissue growth factor 2 (CCN2), a pivotal activator of HSCs, thereby promoting fibrosis [113]. Curiously, CCN2 itself can be present within HSCs-derived EVs, thus enhancing expression of several pro-fibrotic genes in quiescent HSCs, contributing to their activation [114]. On the other hand, EVs secreted by quiescent HSCs were shown to carry high levels of Twist1, a transcription factor that binds to the

miR-214 promoter, increasing its expression. In turn, miR-214 expression is stimulated in receptor cells, thus suppressing the expression CCN2 and its downstream effectors [115].

Current evidence indicates that accumulation of fat in the adipose tissue actively contributes to hepatic steatosis, mainly through the release of adipokines and free fatty acids (FFAs) into circulation, which will lastly end up in the liver [116]. EVs, more specifically exosomes, have already been implicated in this process, representing an important component of the adipose tissue-liver axis. Visceral adipose tissue-derived exosomes obtained from obese patients were shown to dysregulate pro-fibrogenic transforming growth factor-β (TGF-β)-related pathways in both hepatocytes and HSCs [117]. Furthermore, exosomal miRNAs isolated from human and mice adipose tissue constitute the majority of circulating exosomal miRNAs [118]. Of note, under physiologic conditions, exosomal miR-99b targets the hepatic fibroblast growth factor-21 (FGF-21) and represses its expression. Interestingly, only exosomal, but not free miR-99b, is able to regulate FGF-21 in the liver [118]. Finally, transfer of exosomes from adipose-derived stem cells (ADSC) to obese mice improved insulin resistance and reduced obesity, in parallel with reduction of hepatic steatosis. Furthermore, the authors reported that ADSC-derived exosomes induced a M2 anti-inflammatory phenotype when transferred into macrophages, a process that was dependent on the activation of arginase-1 by STAT3-carrying exosomes [119]. In parallel with the adipose tissue-liver axis, several studies support the notion of a gut-liver axis in the pathogenesis of several liver diseases [120]. In fact, high-fat diet-induced dysbiosis in mice leads to the release of gut-derived exosomes carrying injury-related high mobility group box 1 (HMGB1) protein, thus contributing to the development and progression of hepatic steatosis [121]. Last but not least, EVs have also been reported to have pro-angiogenic properties in the liver through targeting of endothelial cells [103, 122]. In particular, hepatocytes undergoing lipoapoptosis release microvesicles exhibiting Vanin-1 on its surface, thus allowing interaction with lipid raft domains of endothelial cells, resulting in cell migration and tube formation [103]. Interestingly, treating primary rat endothelial cells with plasma-derived exosomes from high-fat diet-fed rats markedly increased oxidative stress and the expression of vascular cell adhesion molecule 1 (VCAM1), thus contributing to a pro-inflammatory and pro-angiogenic environment [123]. In addition, exosomes carrying Sonic Hedgehog (Shh) and Indian Hedgehog ligands (Ihh), released by HSCs and cholangiocytes, engage a pro-angiogenic switch in endothelial cells during cirrhosis [122]. However, the role of these EVs in angiogenesis in the NAFLD context needs further exploration.

Extracellular Vesicles as Non-invasive Biomarkers for NAFLD

It is now widely established that circulating EVs are remarkably stable, thus constituting promising non-invasive biomarkers [100]. A pioneer study on this field compared the EV blood profile from patients with simple steatosis (NAFL) or NASH, to

patients with chronic hepatitis C (CHC) or healthy controls. In order to ascertain the possible cells of origin, the authors measured the presence of EVs with leuko-endothelial surface markers by flow cytometry [124]. Serum EVs derived from $CD4^+$ and $CD8^+$ T cells were found increased in patients with NAFL/NASH and CHC, compared to healthy individuals, but they were unable to specifically differentiate between these disease conditions (AUC: 0.57 and 0.65, respectively). On the other hand, patients with NAFL/NASH displayed marked increases in serum EVs containing surface markers from invariant natural killer T cells (iNKT) and monocytes/macrophages ($CD14^+$), as well as lower levels of neutrophil- ($CD15^+$) and endothelial cell- ($CD41^+$) derived EVs. Noteworthy, $CD14^+$- and iNKT-derived EVs positively correlated with serum ALT levels and NAS score, and allowed the differential diagnosis of NAFL/NASH and CHC (AUC:0.999 and 0.97, respectively). Importantly, these two type of EV populations were reported to be key players in liver fibrosis during NAFLD pathogenesis [125, 126]. Further, the release of EVs from immune cells might be involved in liver inflammation and, consequently, in the progression of NAFL to NASH. The levels of adipose tissue-derived EVs in obese patients also correlate with the levels of liver transaminases and were shown to contribute to insulin resistance, interfering with the insulin signaling pathway in hepatocytes [127]. C16:0 ceramide- and S1P-enriched EVs might also embody promising diagnostic biomarkers for NALFD/NASH, since they were shown to progressively increase in the plasma of obese patients with simple steatosis, and further in NASH patients with early fibrosis (F1), when compared with control obese patients [102]. However, these findings were obtained from a small cohort of patients (n = 43) and their diagnostic accuracy remains incompletely explored. As such, further studies, including larger cohorts of patients, should ideally be performed. Specifically concerning fibrosis, a previous study found that $CD14^+$ and $CD16^+$ EVs count could predict fibrosis severity, being inversely associated with NAFLD-related liver fibrosis, while also increasing the diagnostic capability of the enhanced liver fibrosis score (LFS) in patients with NAFLD (AUC: 0.948 and 0.967 for $CD14^+$ and $CD16^+$ EVs, respectively, vs. 0.915 for LFS alone) [128].

Increased concentration of serum EVs has been described in dietary murine models of NASH. In mice fed a choline-deficient L-amino acid defined (CDAA) diet, EV levels were shown to increase early in disease progression, further increasing with time and correlating with hepatocyte cell death, fibrosis and neo-angiogenesis [101]. Furthermore, proteomic analysis of blood EVs from CDAA-fed mice revealed a distinct protein cargo, when compared with EVs isolated from control mice, with most of the identified proteins being already described as players in NASH pathogenesis, namely affecting cell death and inflammatory pathways, among others. Although no AUC values were reported, the authors stated that this proteomic signature allowed for the discrimination of diseased mice compared to controls. In addition, miR-122, a liver-specific miRNA, was found enriched in blood EVs from mice fed a CDAA diet compared to controls, while its hepatic levels were reduced, pinpointing for a potential diagnostic capability of miR-122-EVs for NAFLD [101]. However, future validation studies should be performed, in order to clearly assess its accuracy for NAFLD. Similarly, increased amounts of hepatocyte-derived EVs were detected in blood from diet-induced

NASH mice, and found to correlate with disease severity [103]. In a similar model, endothelial-derived EVs ($CD144^+$) were found increased, an effect that could be reverted by treatment with atorvastatin [129]. EV-derived hepatocyte mtDNA is also increased in the plasma of both mice and human patients with NASH, contributing to the activation of the TLR9 pathway, and the activation of sterile inflammation [107]. Nevertheless, the mechanisms by which mtDNA is targeted in EVs, as well as the accuracy of these particles to diagnose NAFLD remains to be clarified.

Conclusions and Future Directions

In the past decade, the number of reports addressing the role of EVs in human disease has grown exponentially. Particularly for liver diseases, including NAFLD, hundreds of papers have already been published, showcasing promising results that might translate into the clinics in a near future. Nonetheless, several aspects still need to be addressed before this jump can be made, particularly those related with methodological aspects. For instance, many studies use the terms "exosome" and "microvesicles" indiscriminately, without proper characterization of the isolated EV fraction. Further, considering that EV biogenesis is not entirely understood, and purification protocols are not homogeneous, many inconsistencies are still found in the literature [79, 80, 85]. In order to bypass these problems, and aid in the rapid translation of EVs into the clinics, standardized, large-scale and cost-effective protocols are urgently needed. In this regard, the EV-TRACK knowledgebase constitutes a key resource that researchers should consult in order to standardize research in this filed and increase reproducibility of EV-related reports [80].

NAFLD is a complex metabolic multisystem and multicellular disease, involving extra-hepatic organs and several cell types in liver, which encompasses different degrees of autocrine, paracrine and endocrine communication. Although the investigation on this filed is still scarce and inconclusive, there is still much to discover, it is now clear that inter-cell and inter-organ communication in NAFLD might be of pivotal important and is mediated in part, by circulating EVs. Mostly due to the accumulation of toxic lipid species within hepatocytes, these liver cells are currently considered as one of the major sources of EVs in NAFLD, consubstantiating a key mechanism for disease progression into more nefarious stages. Although the role of EVs in NAFLD pathogenesis is unquestionable, there are still unsolved questions that should be addressed in the future: what are the major EV contents that directly contributes for disease progression? And how can we specifically target these EVs? Furthermore, deeply studying the molecular mechanisms underlying EV biogenesis will contribute with key concepts that will hopefully allow the manipulation of EV generation in patients, thus opening a new window for therapeutic interventions. Still, this idea should be approached with caution, as manipulation of the machinery involved in EV biogenesis might hold potential secondary effects on healthy tissues [80]. In this regard, the role of EVs as biomarkers for NAFLD is probably more close to make this translational jump. In fact, many reports have already illustrated the

potential of these vesicles to act as either diagnostic or prognostic biomarkers. In order to advance the field, future studies should assess the diagnostic value of EVs in larger cohorts of patients, including properly characterized individuals (biopsy-proven). Further, stratifying patients according to their metabolic status (presence/absence of diabetes, obesity, hypercholesterolemia, among others), as well as to the presence/absence of fibrosis, might reveal interesting and could provide decisive results. Lastly, it is widely known that patients with advanced NASH are at higher risk of progressing to HCC. It will also be important to query whether EVs might help in in the prediction of patients who might be at risk for experiencing disease progression to HCC. In this regard, we have recently described a specific proteomic profile in serum EVs that allowed the specific diagnosis of HCC, when compared with healthy controls or patients with intrahepatic cholangiocarcinoma [130]. It will now be imperative to conduct studies in order to address the diagnostic capacity of these EVs, in this context.

In the next decade, several EV-related studies are envisioned in the NAFLD field, which might contribute with new concepts that will help in deciphering disease pathogenesis and possibly provide new diagnostic and prognostic tools to be applied into daily clinics [81, 85, 131]. The wide-range cellular and biological functions of EVs, as well as their ability of encapsulating and protecting biological and artificial therapeutic compounds, support the idea that EVs and their components may also be used as novel therapeutic targets, therapeutic agents and/or drug delivery vehicles to treat NAFLD.

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Chapter 10
From Transcriptomic to Metabolomic in the Development of Biomarkers in NAFLD/NASH

George V. Dedoussis and Athina I. Amanatidou

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide [1]. The health and socioeconomic impact of NAFLD/NASH is grown increasingly with the passage of time and the annual medical costs are higher than $103 billion in the United States [2]. Therefore, its early and accurate diagnosis is quite important given the fact that its prevalence has rapidly reached global epidemic rates, both in children and adults [3]. Unfortunately, most of the patients do not develop symptoms, so the disease is considered to be asymptomatic and in most cases the diagnosis is random [4].

Growing evidence suggests that the onset of the disease is due to a complex process in which many factors are involved [5, 6]. Over the last several decades, the scientific community has focused on the causes of the disease and the discovery of novel diagnostic markers (*biomarkers*). Nevertheless, the gold standard for NAFLD/NASH diagnosis remains the liver biopsy [7] but this method is impractical as a diagnostic tool due to it being invasive, costly and occasionally associated with severe complications (e.g. pain, serious bleeding, infection and sampling errors). Evidently, a liver biopsy is an unrealistic method to be applied on each NAFLD patient, given that the latter affects more than a quarter of the whole population. In the near future—as it is expected—the "molecular signature" of each NAFLD patient could be the key for their diagnosis and treatment. The data that derived from omics technologies which feed precision medicine have a major contribution to this effort.

Precision medicine [8]—also known as *personalized medicine*—is defined as: "The tailoring of medical treatment to the individual characteristics of each

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patient…to classify individuals into subpopulations that differ in their susceptibility to a particular disease or their response to a specific treatment. Preventative of therapeutic interventions can then be concentrated on those who will benefit, sparing expense and side effects for those who will not". According to the definition, precision medicine has the power to enhance the guidance of health care decision for a given patient and as a result be the leader of improvement of care quality with no need for pointless diagnosis tests and treatments.

In the last decades, there are ever-increasing data of omics technologies which feed precision medicine. The aim of such high-throughput technologies is the collection of genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolite (metabolomics) in a given biological sample. In the main text of this chapter, we will emphasize on the pathway from transcriptomics to metabolomics in the development of biomarkers in NAFLD/NASH. To our knowledge, *transcriptomics* include the full set of mRNA within a cell, tissue or organism; *proteomics* comprise the study of a large set of proteins, involving their structure and functions, which are present in a cell or tissue type and *metabolomics* is the large-scale study of metabolite profiles within cell, tissue or organism under a given set of conditions [9]. However, the omics technologies do not contribute directly to precision medicine, but via the detection of biomarkers [9], which are generated from the systemic analysis of omics. The question which reasonably arises is whether all of this information is ready to be implemented in clinic practice.

As referred previously, the nature of NAFLD is a complex multifactorial process. Recently, the "multiple parallel hits hypothesis" [10] rather than "first and two-hit hypothesis" dominated to define development of NAFLD. The rapid growth of omics gives an impetus to many scientists to further study NAFLD, thusly allows for scientific knowledge to gain ground. Apart from essential role of genomic studies for the identification of genetic variations associated with NAFLD/NASH, the contribution of transcriptomics, proteomics and metabolomics studies is equally important. The profiles derived from transcriptomic alterations, over or under-expressed proteins and plasma metabolics provide biomarkers which are associated with the characteristics of NAFLD/NASH. This chapter aims to review the newly discovered biomarkers resulting from these studies.

The '-Omic Pathway' in the Development of Biomarkers in NAFLD/NASH

Transcriptomics in NAFLD/NASH

The transcriptomic analysis enables researchers to detect global mRNA expression changes in NAFLD. Several studies have been conducted in this field; the most recent literature may be found below.

Many attempts have been made (Suppli et al. [11], Pettinelli et al. [12], Zhang et al. [13] and Arendt et al. [14]) in order to elucidate insights into pathology of

NAFLD. Concerns have arisen that the existing diagnostic tools are unable to differentiate mild NAFLD from NASH, demonstrating that more histological markers are considered necessary. More recent evidence (Suppli et al. [11]) suggests that the utility of applying immunohistochemical markers of hepatocyte injury may provide a more objective diagnostic tool for the discrimination of NASH from NAFL. In the aforementioned study, global gene expression was evaluated in 31 NAFLD patients (16 of whom were NASH) and compared to the one of 14 healthy normal-weight and 12 obese subjects. The results showed that NASH patients with positive Sonic hedgehog hepatocyte in (SHH) staining had differential gene expression in comparison with NAFL patients. Several of the genes that were found differentially expressed (CAPN2, COL1A2, COL4A2, EDA, ELK1, CASP1, STMN2 etc.), are implicated in extracellular matrix organization and remodeling, cell adhesion, cell cycle control, apoptosis, metallothionein-family antioxidant proteins, and microtubule dynamics. Importantly, a total of 132 genes (112 of which are upregulated and 20 are downregulated) were found significantly regulated in NASH versus NAFL. Amongst the top ten genes that were significantly upregulated are MMP2 (matrix metallopeptidase 2), FMNL3 (formin like 3), OTOA (otoancorin) etc. Similarly, some of the downregulated genes are the following; SLC25A48 (solute carrier family 25 member 48), MT1E (metallothionein 1E), MAT1A (methionine adenosyltransferase 1A) etc.

In the cross-sectional study of Pettinelli et al. [12], the hepatic gene expression was assessed in 17 individuals with simple steatosis (SS), 15 with NASH and 22 controls derived from living liver donors (LD). They reached the conclusion that the hepatic gene expression of AKR1B10 is highly upregulated in NASH individuals compared to SS and LD. Also, they underlined the fact that the overexpression of AKR1B10 induces the reduction of hepatic retinaldehyde levels, with the subsequent decreasing of retinoic acid which is may be a key for the progression of NASH to hepatocellular carcinoma (HCC). In accordance with their findings, the expression of AKR1B10 was upregulated between NASH compared to SS and LD individuals. Additionally, they suggested that the overexpression of AKR1B10—which induces the underexpression of ALDH1A2 and ALDH1A3—could be a potential contributing factor to the progression of NASH to HCC.

In the last few years, the interest of scientific community is oriented on the study of long non-coding RNAs (lncRNAs) (non-coding transcript larger than 200 nucleotides) regarding the development or progression of NAFLD fibrosis [15]. To date, more studies of lncRNAs have been conducted in animal than in humans with NAFLD fibrosis. Remarkably, there are indications that these molecules are key contributors to biological processes, playing an important role of the pathophysiological mechanisms underlying the disease. More recent research by Atanasovska and colleagues (2017) [15] reveals that lnc18q22.2 showed elevated expression levels in liver tissue of NASH patients. Additionally, they proposed that this lncRNA can play a significant role in regulation of liver function—bringing alternative perspectives to the regulation of hepatocyte viability in NASH.

Recently, microarrays allowed the identification of microRNAs (miRNAs) in NAFLD. miRNAs are non-coding short RNA molecules which are consisted of

19–23 nucleotides, regulating the stability of mRNA and thus the transcription levels which affect the development of NAFLD [16]. Importantly, the stability of miRNAs makes them stable in clinical samples. Hence, circulating miRNAs (miRNAs detected in cell-free or plasma) have been recommended as a non-invasive diagnostic tool for discriminating NAFLD from healthy individuals. Their effectiveness results from their capacity to reflect the physiological or pathological state of the organ/tissue that they are derived from. Pirola et al. [16] studied the expression of 84 circulating miRNAs in biopsy-proven NAFLD patients and healthy individuals, which led to the finding that 6 miRNAs are upregulated. The authors focused their interest on miR-122 and miR-192, which have the most significant fold changes that were found in the serum levels of patients. Based on their observations, it was concluded that miR-122 has a significant role in pathophysiology of NAFLD.

Proteomics in NAFLD/NASH

It has become feasible, as proteomic technologies advances, the detection of protein biomarkers—key molecules that reflect the biological pathways underlying disease. The application of proteomics in the study of NAFLD/NASH holds a great potential in providing important insights into its pathogenesis, as well as discovering novel biomarkers. It is widely accepted that the quantification of protein's expression levels from a given biological sample (e.g. serum, plasma) and their subsequent analysis, allows the identification of proteomic profiles, that one may be used as a diagnostic fingerprint in NAFLD.

Since 2005, much more information on the investigation of protein biomarkers in NAFLD had made available via proteomics analysis. The proteomic profiles in serum of NAFLD patients were firstly investigated in the study of Younossi et al. [17], in which 12 proteins was found with significant differential expression. Due to the limitations of the aforesaid study, eventually the authors stood only in the fibrinogen γ as a candidate associated factor with fibrosis.

Given the fact that the majority of blood plasma proteins are secreted in the liver, it was therefore necessary for researchers to investigate the plasma proteome which affected in the case of liver disease. For this reason, more recently, the study of Niu et al. [18] focused on the investigation of plasma proteome profiling of 48 NAFLD participants. The results obtained in the abovementioned study reported six proteins that were found statistically significant dysregulated. The resulting proteins are the fructose-bisphosphate aldolase B (ALDOB), apolipoprotein M (APOM), galectin-3 binding protein (LGALS3BP), polymeric immunoglobulin receptor (PIGR), vitronectin (VTN) and afamin precursor (AFM).

It is widely acknowledged that the human metabolic disorders (obesity or diabetes) are related to abnormal liver function and thus to NAFLD. Reasonably, the

latest studies uncover the existence of smaller high-density lipoprotein (HDL) particles in NAFLD patients when compared to individuals lacking liver abnormalities. Alterations of HDL particles—either in their size or protein composition—have been highlighted in metabolic disorders leading to dysfunction. Also, these alterations have been identified to associate with increased cardiovascular disease (CVD) risk. The "knowledge gap" that until recently had not been addressed was the assessment of protein composition of HDL particles in NAFLD patients and by extension their relation to CVD risk.

The study of Rao et al. [19] was the first proteomic analysis in an attempt to identify proteomic alterations of HDL particles in a group of 15 morbidly obese females consisting of 5 with SS, 5 with NASH and 5 with normal liver histology, with the aim of preventing the CVD risk in such patients in the future. In accordance with their proteomic analysis, the outcome revealed 95 HDL-associated proteins that were found with quantitative differences between SS, NASH and normal subjects. Nevertheless, 12 of 95 proteins have found to be present with nominally significant differences—those with the most statistically significance (p-value < 0.01) are the following: alpha-2-macroglobulin (A2MG), alpha-1-antichymotrypsin (AACT), antithrombin III (ANT3) and corticosteroid binding globulin (CBG). The abovementioned study concluded that the alterations of HDL proteome may affect the HDL particles' function in NAFLD/NASH, possibly causing an increased CVD risk in these patients.

Another aspect of research in NAFLD pathogenesis that is equally important is the study of lipid droplets (LDs), as this disease is characterized by excessive accumulation of LDs in the liver. Moreover, LD has shown to be associated with several clinical outcomes such as metabolic disorders, hepatosteatosis and CVD risk. Interestingly, the study of Su et al. [20] deals with LDs which were obtained from 21 human liver biopsies, consisting of 12 individuals with SS (cases) and 9 individuals with normal liver histology (controls). Subsequently, proteomic analysis was conducted in order to proceed with the comparison of proteome LDs profiling amongst the aforementioned cases and controls, providing proteins that were differentially expressed. Finally, their study resulted in the following findings; specifically in the identification of 89 dysregulated LD-associated proteins in NAFLD, 54 of which were found to be upregulated and the remaining downregulated. The perilipin, ADRP and TIP47 (PAT family members) are detected amongst the upregulated proteins, enhancing the claim that the elevated expression of this family members may play a part in accumulation of LDs in fatty liver. The finding that stood out most was the discovery of 17β-hydroxysteroid dehydrogenase-13 (17β-HSD13) as a new LD-associated protein, which may be involved in NAFLD pathogenesis. They further validated the proteomic findings of 17β-HSD13 in mice model, confirming its involvement in hepatic lipogenesis in normal mouse liver. Thus, 17β-HSD13 could be considered as a candidate therapeutic target of fatty liver disease.

Metabolomics in NAFLD/NASH

In recent years, a great deal of scientific attention has been devoted to metabolomics. Through the era of metabolomics, scientists have the opportunity to identify hundreds of metabolites characteristics in many complex diseases. In case of NAFLD, metabolomics is a powerful weapon for the evaluation of liver injury, given the fact that the most commonly sample used for testing is urine or serum. Several studies have been conducted in order to identify differences of metabolite profiles in NAFLD patients, aiming to provide the detection of serum and urine metabolite biomarkers within a dynamic field. These biomarkers seem to be effective in diagnostic practice either to differentiate the stages of NAFLD or to indicate a predisposing to the NAFLD development.

The importance of revealing the molecular mechanism behind the progress from NASH to NAFLD is critical. Dong and colleagues (2017) [21] in an effort to reach the abovementioned goal, they performed metabolomics analysis to ascertain changes in metabolic profiles amongst NAFLD and NASH subjects. The urine and blood samples were collected from 33 patients with NAFLD (with normal liver function), from 45 with NASH (with abnormal liver function) and from 30 healthy individuals. After comparing differences of urinary metabolomics between NAFLD and NASH groups, 32 metabolites were highlighted, including nucleic and amino acids. According to ROC (receiving operating characteristic) analysis, they concluded that the metabolites which most distinguished NASH from NAFLD are the indolelactic acid, 3-indoleacetic acid, L-carnitine and pyroglutamic acid. Additionally, the clinical trials of this study showed metabolic alterations between NAFLD and control group; these are the concentrations of serum glucose, glutamate, lactate and taurine.

Although a great number of serum biomarkers have been proposed by various studies either for the differentiation of normal liver (NL) and NAFLD or NASH from NAFLD, however, no one of them had proven to be successfully applied in a large cohort of biopsy-proven NAFLD patients. Also, their further validation in an independent blind cohort is still lacking. More recently, Mayo et al. [22] aimed to uncover more reliable, accurate and robust serum biomarkers that could be have an effective role in aforementioned discrimination of diseases. For this purpose, they developed a metabolomics study enrolling 467 biopsy-proven NAFLD patients (246 with NAFL, 131 with NASH, and 90 with NL) for the detection of serum lipidomic profiling. The resulting biomarkers are later confirmed in an independent group of 192 biopsy-proven NAFLD patients (109 with NAFL, 76 with NASH, and 7 with NL). They demonstrated a panel of total 28 triglycerides (TGs); more specifically 11 TGs for the discrimination between NL and NAFLD, as well as 20 TGs for NASH and NAFL. Their study contains some strong aspects; the high sensitivity and specificity of applied lipidomic tests both in initial and validation groups within a large enough sample size.

Remarkably, Papandreou et al. [23] investigated the rearrangement of lipid biosynthesis applying in a total of 45 individuals and after a mean follow-up of 3.8 years. This studied group classified into three subgroups in accordance with

the hepatic steatosis index. Each category consisted of 15 participants; (1) cases non-characterized as NAFLD, (2) cases characterized as NAFLD, and (3) cases characterized as NAFLD-reversion. The authors observed, among others, that the rearrangement of lipid biosynthesis could be affected by the decreased levels of glycerophosphocholines, ceramides and sphingomyelinase. Interestingly, they proposed that the studied individuals may have an immune dysfunction; given the increased plasma levels of L-cystine/L-glutamine ratio which were found to be positively correlated with circulating levels of TNF-alpha. Interestingly, they concluded that the liver or serum of NAFLD patients could be affected by a rearrangement of lipid biosynthesis, making it up a prognostic factor of NAFLD development.

As is well known, a dysregulated metabolism of branched-chain amino acids (BCAAs) is correlated with NAFLD. Several studies suggest that these alterations may lead to the development of insulin resistance, which had been show to be closely associated with NAFLD in adult patients. Nevertheless, the existing adolescent studies reported conflicting results. The study of Goffredo et al. [24] in an effort to analyze a metabolomics signature in 78 obese adolescents with (n = 30) vs. without (n = 48) NAFLD, reached into interesting results as mentioned below. After evaluating 180 plasma proteins in these adolescents; plasma levels of valine, isoleucine, tryptophan, and lysine were detected elevated in NAFLD adolescents. Furthermore, an increscent of baseline valine levels was proposed to be an indicative factor of major hepatic fat accumulation.

A Perspective of Systems Biology in NAFLD/NASH

As mentioned above, NAFLD—as many other complex diseases—is multifactorial in nature and several complex processes trigger its development and advancement. Up to date, an increasing number of technological advancements provide a collection of many unused data as a whole. The transition from single-omics to multi-omics analysis is therefore necessary, offering a wider window of its pathophysiology—scanning different point of views.

Systems biology [25], a computational branch of biology, is based on assumption that the study of the whole system of living organism is more powerful than its individual parts. It integrates a collaboration of multiple scientific disciplines, such as bioinformatics, biology, computer science, physics and etc., dealing with a dynamic biological system in which its behavior becomes predictable and depends on changing conditions. Thus, a construction of predictive multiscale models facilitates the researchers to uncover novel disease biomarkers and to design therapeutic strategies.

Systems biology tools have been developed, integrating omics data. These tools are extensively used by researchers to reveal the causes behind human diseases [26]. The research of NAFLD includes, for the most part, human clinical and animal models studies. The application of novel systems biology strategies could be

beneficial in the elucidation of NAFLD pathophysiology and treatment, contributing to the development of noninvasive diagnosis.

It is worthwhile noting that systemic biologic approaches were applied by the study of Sookoian et al. [27] so as to clarify the common pathogenic pathway between alcoholic fatty liver disease (AFLD) and NAFLD. The integration of omics and physiological data was accomplished, and their subsequently analysis was performed using gene enrichment analysis and protein-protein interaction network. According to analysis, they reached a conclusion that NAFLD and AFLD share similar disease mechanisms, but different molecular signatures are characterized them. Moreover, the authors showed that only NAFLD is involved in cardiovascular disease and the insulin signaling is blocked in the state of fatty liver.

A Window into the Future

With the passage of time, NAFLD is rapidly gaining ground in the scientific community. NAFLD is a serious disease and its global prevalence is growing. Knowledge gained on transcriptomics, proteomics and metabolomics signatures in NAFLD and NASH, should be validated and then to be implement into the clinical practice. Effective preventative and therapeutic strategies are in need to form so as to stop the rapid growth and development of NAFLD. Pathophysiology context of NAFLD can be varying from one patient to another, resulting in the onset of disease. Hence, the heterogeneous nature of NAFLD demands a personalized approach of patient-health care by their own "molecular signature." In the future, the personalized diagnosis could be a combination of all data gathered from omics technologies and patient's genetic make-up. This leads to personalized drugs and treatment; where should be made according to a given "molecular signature". The personalized diagnosis could enhance our scientific knowledge behind disease biology, allowing targeted medical interventions. Furthermore, a more detailed personalized lifestyle intervention is equally important, besides the well-known basic recommendations like healthy diet, alcohol restriction and exercise.

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Chapter 11
HCC in Patients with NAFLD/NASH

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Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignant tumour of the liver and one of the most prevalent neoplasms in the world [1–3]. Together with hepatitis B virus (HBV), hepatitis C virus (HCV), prolonged long-term alcohol consumption, aflatoxin, and metabolic liver disease, non-alcoholic fatty liver disease (NAFLD) is a significant risk factor for HCC. Since the first reported case of HCC associated with NAFLD in 1990 [4], the number of cases has been steadily increasing [5]. In the last years, Wong et al. demonstrated in two studies that non-alcoholic steatohepatitis (NASH) is the most rapidly growing risk for liver transplantation in patients with HCC [6, 7].

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In the vast majority of cases, HCC develops in the context of hepatic cirrhosis, but recent data suggest that there is a proportion of patients with NAFLD who are at high risk for HCC in the absence of cirrhosis [8–11]. In 2009, our group described seven cases of HCC in histologically confirmed NASH patients. Of these, one was non-cirrhotic (NASH stage 1), demonstrating that relevant fibrosis or cirrhosis is not mandatory for the onset of HCC [12]. Afterwards, in another study by our group with a larger number of patients (42 HCC cases secondary to NAFLD or cryptogenic cirrhosis), we identified almost 10% non-cirrhotic patients in the study [13]. Currently, some studies have demonstrated the onset of HCC in the absence of cirrhosis [13–16]. In a multicentre national Brazilian study involving nine hepatology units from six states of the country, we evaluated the clinical characteristics of 110 patients diagnosed with HCC and NAFLD. In this study, the mean age was 67 ± 11 years and 65.5% were male. Obesity was observed in 52.7% of the cases, type 2 diabetes mellitus (T2DM) in 73.6%, dyslipidaemia in 41%, arterial hypertension in 60%, and metabolic syndrome (MtS) in 57.2%. The histological diagnosis of HCC was made in 47.2% of the patients, and among these patients, NASH with cirrhosis was observed in 61.5%, NASH with fibrosis stage 1–3 in 27%, and NAFLD without fibrosis in 3.8% [17]. Evidence that HCC is part of the natural history of NASH comes from retrospective studies, case reports, and studies evaluating the late complications of NASH and cirrhosis associated with NASH [18].

Pathophysiology of NASH-Related HCC

Although no study clearly explains the evolution of NAFLD to HCC, researchers suggest that the most likely mechanism involves fatty acid (FA) accumulation in hepatocytes due to predisposing factors, such as MtS, increased oxidative stress, endoplasmic reticulum (ER) stress, mitochondrial dysfunction and chronic endotoxaemia. All these phenomena contribute to the onset of inflammation and fibrosis, leading to a chronic and progressive lesion [19, 20].

Studies show that 70% of individuals with T2DM and 90% of individuals who are obese have some form of triglyceride (TG) accumulation in the liver, such as simple steatosis (60%), NASH (25–30%) or cirrhosis (5–10%), indicating that NAFLD and NASH are common in these individuals. NAFLD and NASH may be the link between MtS (with obesity as the main factor) and HCC, that is, individuals who have the main factors of MtS [insulin resistance (IR)/T2DM and obesity] present with high chances of TG accumulation in the liver, which in turn can lead to NAFLD and progress to NASH, cirrhosis and HCC [21–23].

Park et al. (2010) described the development of HCC in the context of obesity as a function of increased expression of tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6). These cytokines, in addition to causing hepatic inflammation, activate the oncogenic signal transducers and activators of transcription 3 (STAT3), which increases the proliferation and progression of hepatocytes with oncogenic mutations, leading to tumour development [24].

Deregulated cell growth in the context of hyperinsulinaemia has been described as a function of insulin-like growth factor 1 (IGF-1), mannose 6-phosphate receptor/insulin-like growth factor receptor 2 (M6P/IGF2R), and the first insulin receptor substrate (IRS-1). Hyperinsulinaemia increases the production of IGF-1, a hormonal peptide that stimulates cell growth by cell proliferation and inhibition of apoptosis in the liver. Insulin also activates IRS-1, which is involved in cytokine signalling pathways and is increased in HCC. MP6/IGF2R, a tumour suppressor gene, regulates cell growth through inhibition of cell proliferation and apoptosis via transforming growth factor beta (TGF-β) and insulin-like growth factor 2 (IGF-2), respectively. Interestingly, MP6/IGF2R mutations have been identified in HCC, even in the absence of viral hepatitis or liver cirrhosis. Adiponectin is an adipose tissue specific anti-inflammatory polypeptide that is decreased in the state of IR and has been shown to be responsible for the inhibition of angiogenesis via modulation of apoptosis in animal models [25, 26].

The development of NASH is also related to oxidative stress and the release of reactive oxygen species (ROS), which probably contribute to the development of HCC. Oxidative stress may favour tumourigenesis through steatosis, inflammation and cell proliferation or may induce mutations directly associated with cancer. It has been shown that 4-hydroxynonenal, a lipid peroxidation product, causes mutations in the p53 tumour suppressor gene that is associated with more than half of human cancers, including HCC [27]. Nuclear factor transcription factor respiratory factor 1 (Nrf1) is essential in the mediation of oxidative stress. Xu et al. (2005) demonstrated in an animal model that the lack of this factor increases the susceptibility to oxidative stress. The hepatic histology of Nrf1-deficient animals showed steatosis, apoptosis, necrosis, inflammation and fibrosis, and the development of HCC related to oxidative stress was observed in some cases [28].

Recently, the c-Jun amino-terminal kinase 1 protein (JNK1) has been related to obesity, IR, NASH and HCC. FA, TNF-α and ROS released in the hyperinsulinaemic scenario are all potent activators of JNK1, which in turn phosphorylates IRS-1. Obesity is associated with abnormal elevation of JNK1 activity. Activation of JNK1 and subsequent phosphorylation of IRS-1 are crucial components of the biochemical signalling responsible for obesity induced by IR. The activity of JNK1 has previously been associated with a variety of cancers. More recently, definitive evidence has demonstrated a significant relationship between sustained activation of JNK1 and development of HCC [25, 29].

Hepatic carcinogenesis in NASH may also be mediated by cellular mechanisms. Hepatocyte injury related to NAFLD leads to an overactivation of the Hedgehog pathway, a complex cellular pathway for repair and tissue regeneration. One of the key mechanisms activated through stimulation of this pathway involves mobilization of hepatic progenitor cell populations to replace damaged hepatocytes. Although essential for liver repair, aberrations in the activation of the hepatic progenitor cell population can lead to impaired repair and dysregulated proliferation of hepatocytes, potentiating carcinogenesis [26, 30].

The role of the adaptive immune system has been recognized through studies in animal models of NASH-related HCC. Factors that activate inflammation such as

nuclear factor kappa B (NF-kB) and insulin receptor (IR) contribute to carcinogenesis in patients with NAFLD. Ma et al. (2016) demonstrated in an animal model that the deregulation of lipid metabolism in NAFLD leads to a selective loss of CD4+ T lymphocytes but not CD8+ T lymphocytes, leading to increased hepatic carcinogenesis [31]. In another study by Wolf et al. (2014), which also used an animal model, described the metabolic activation of CD8 + T lymphocytes and natural killer (NK) cells acting synergistically with inflammatory cytokines and leading to liver damage and inducing carcinogenesis [32].

Another mechanism that has been associated with the pathogenesis of HCC secondary to NAFLD is genetic predisposition. Romeo et al. (2008) described a single nucleotide polymorphism (SNP) in the Patatin-like phospholipase domain containing 3 (PNPLA3) gene, which strongly affects the accumulation of fat in the liver in the absence of IR. The PNPLA3 risk allele rs738409 [G] is found in approximately 40% of the European population and may increase the risk of progression to NASH threefold and, more importantly, the risk of developing HCC 12-fold [33].

The Fig. 11.1 summarizes the possible mechanisms involved in the evolution/progression of NAFLD to HCC.

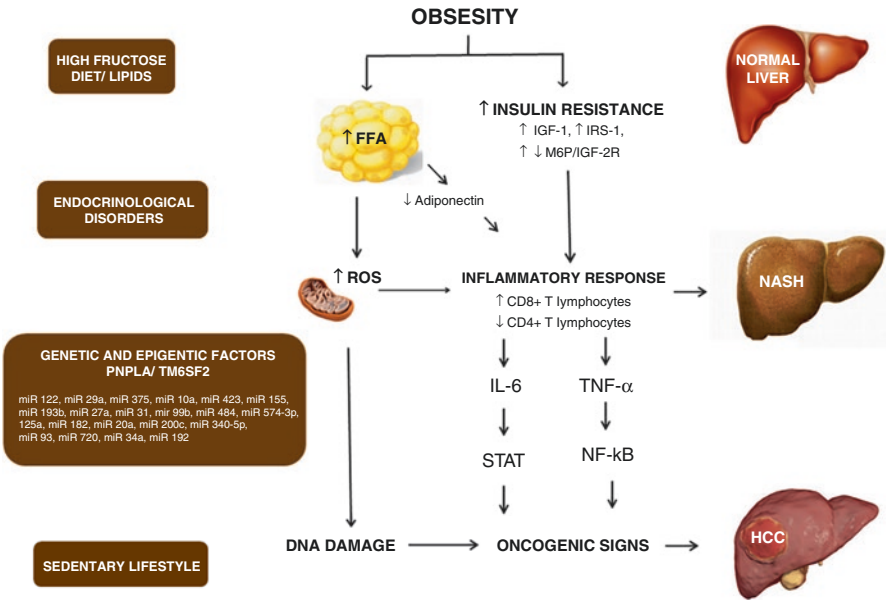

Fig. 11.1 Possible mechanisms involved in the evolution/progression of non-alcoholic fatty liver disease (NAFLD) to hepatocellular carcinoma (HCC). Legend: *NASH* non-alcoholic steatohepatitis, *HCC* hepatocellular carcinoma, *FFA* free fatty acids, *ROS* reactive oxygen species, *IGF-1* insulin-like growth factor 1, *IRS-1* insulin receptor substrate 1, *M6P/IGF2R* mannose 6-phosphate receptor/insulin-like growth factor receptor 2, *TNF-α* tumor necrosis factor alpha, *NF-kB* nuclear factor kappa B, *IL-6* interleukin-6, *STAT* signal transducers and activators of transcription

Biomarkers in NAFLD Progression and HCC

Genetic and epigenetic changes in pathogenesis and hepatic carcinogenesis potentially serve as potential diagnostic biomarkers and/or therapeutic targets. MicroRNAs (miRNAs) play a key role in the pathogenesis of HCC related to NAFLD by closely regulating lipid metabolism, glucose homeostasis, cell proliferation, apoptosis, and cell migration and differentiation [34]. Due to the effect of miRNAs on lipid metabolism and hepatic carcinogenesis, miRNAs have been considered a novel therapeutic target for metabolic disorders and HCC. Maintaining normal lipid and glucose homeostasis may prevent the development of HCC related to NAFLD. However, there is still a long way to understand the comprehensive molecular mechanisms of pathogenesis and progression of NAFLD-related HCC.

Similar to other metabolic diseases, NAFLD is a multifactorial disease in which genetic predisposition, environmental exposures, and lifestyle factors can modulate susceptibility to the disease and its progression [35]. Thus, there is a strong interest in identifying biomarkers to detect NAFLD early and monitor the progression of the disease.

Studies have shown that non-coding RNAs (ncRNAs) are implicated in the aetiology of NAFLD and are possibly the main mediators in their pathogenesis [36, 37]. ncRNAs are constitutively expressed and can regulate biological processes, genes and proteins [38]. miRNAs are the most studied ncRNAs with their well-defined biogenesis and processing [39]. miRNAs are highly conserved single-stranded short-chain ncRNAs (~18–22 nucleotides) that can regulate gene expression via specific complementary binding to target mRNA and result in degradation of mRNA (or perfect binding) or translational deletion (imperfect binding), although such silencing of mRNA expression can be reversed [40]. In addition, miRNAs are involved in post-transcriptional regulation associated with cell proliferation, differentiation and death and carcinogenesis. Circulating miRNAs are stable in body fluids, and serum levels of some miRNAs are altered under certain pathophysiological conditions, which make them excellent non-invasive biomarkers for monitoring the progression of liver disease. As a result, they can identify early and non-invasive patients at greater risk.

miR-122 is a liver-specific miRNA found in the circulation, especially in exosomes, when there is liver damage. A recent study has demonstrated that miR-122 expression is increased in NASH patients compared to patients with simple steatosis and a close correlation between miR-122 concentration and histological degree of disease severity [41]. The authors have also shown an association between serum levels of miR-122 and NAFLD Activity Score (NAS) in addition to the presence of hepatocyte balloonisation and advanced fibrosis as well as a strong correlation between miR-122 and liver enzymes [41]. miR-122 has been shown to be a better biomarker than Cytokeratin 18 (CK-18), aspartate aminotransferase (AST), or alanine aminotransferase (ALT), (biomarkers used in clinical practice to indicate presence of liver disease) to predict patients with NASH and fibrosis in this population. However, this difference in transaminases is subtle, and at NAS scores above 5,

miR-122 and hepatic enzymes have the same predictive capacity [34]. On the other hand, few studies have demonstrated the role of miR-122 expression in HCC from NASH patients. Some years ago, Takaki et al. (2014) suggested experimentally and in liver tissue that silencing of miR-122 is an early event during hepatic carcinogenesis related to NASH, and miR-122 could be a novel molecular marker for evaluating the risk of HCC in patients with NASH [42]. More recently, in NAFLD patients, Akuta et al. (2016) demonstrated that HCC and/or histological components of NASH affected serum miR-122 levels independently. In longitudinal evaluations by these authors, serum miR-122 levels had already tended to decrease before the progression of the fibrosis stage [43]. However, on the contrary, our group studying 84 miRNAs expression using Liver miFinder miRNA PCR Array (QIAGEN) in all stages of NAFLD progression, including NASH/HCC (n = 5), identified higher levels of miR-122-5p in the progression of NAFLD, including NASH/HCC compared to other patients, suggesting that these miRNAs were modulated during transition from NASH to HCC. In addition, we identified in this study that miR-375 and miR-29a-3p are associated with miR-122-5p as novel molecular pathways for therapeutic intervention (unpublished data). Interestingly and similarly to our study, An et al. (2018) studying 84 cases of HCC from diverse causes identified that miR-375, miR-10a, miR-122 and miR-423 were significantly higher in HCC than in controls and suggested a novel serum microRNA panel for the diagnostic and prognostic implications of hepatocellular carcinoma [44].

Recently, miR-155, which is a direct regulator of the inflammatory cytokine TNF-α, has been associated with inflammation. miRNA-155 has important function in the early stages of choline-deficient and amino-defined (CDAA) hepatic carcinogenesis. Wang et al., 2009 demonstrated that the activation of CDAA-induced NF-κB increased the hepatic expression of miRNA-155, resulting in the induction of growth of Hep3B and HepG2 cells and the depletion of endogenous miRNA-155 inhibited cell growth SNU-182. Recently, Tessitore et al. (2016) described in animal a model of HCC-NASH, nine overexpressed miRNAs in tumours (miR-155, miR-193b, miR-27a, miR-31, miR-99b, miR-484, miR-574-3p, miR-125a-5p, and miR-182) and five downregulated miRNAs (miR-20a, miR-200c, miR-93, miR-340-5p, and miR-720) with respect to non-tumour tissues [45]. Interestingly, another miRNA, miR-375, is a key molecule for the regulation of glycaemic homeostasis and can inhibit glucose-induced insulin secretion, while elimination of miR-375 may increase insulin secretion [41]. A study by Pirola et al. (2015) demonstrated overexpression of miR-375, which was significantly associated with degree of histological severity, with degree of cellular balloonization and NAS [41].

Other miRNAs have been described in NAFLD. miR-34a is one of the most lipid-related liver miRNAs and its expression is closely related to the severity of NASH, since its overexpression results in apoptosis of hepatocytes. The main target of miR-34a is the NAD-dependent deacetylase Sirtuin-1 (SIRT1), which plays a key role in energy homeostasis by activating important transcription factors such as peroxisome proliferator-activated receptor alpha (PPARα) and liver X receptor (LXR). The silencing of miR-34a promotes the expression of SIRT1 and PPARα, resulting in the activation of AMP-activated protein kinase (AMPK) and the activa-

tion of numerous target genes of PPARα, suggesting that miR-34a functions in the deregulation of lipid metabolism associated with NAFLD [46]. Although it is not known exactly how the mechanism of action of miR-34a influences the development of NAFLD, studies have shown its dysregulation in this disease. In 2016, Xiao-Lin Liu et al. analysed the viability of miR-34a as a diagnostic marker of NASH. In this study, they identified a correlation between the serum concentrations of miR-34a, miR-122 and miR-192 and hepatic steatosis and inflammatory activity. In addition, miR-34a presented a higher correlation with lobular inflammation, and miR-122 presented a greater association with hepatocellular balloonization [47]. However, Bharali et al. (2018) demonstrated an inverse correlation between HCC expression and miR-34; nevertheless, the HCC samples were from hepatitis B [48]. Additionally, Zhou et al. (2018) demonstrated in HCC tissues and cells that miR-34a inhibits HCC progression by repressing hexokinase-1 [49]. Nevertheless, there are few studies on HCC secondary to NAFLD.

Recently, in high fat diet (HFD)-treated mice and human HepG2 cells incubated with FA, Wu et al. (2016) identified a novel mechanism by which miR-21, in part, promotes hepatic lipid accumulation and cancer progression by interacting with the Hbp1-p53-Srebp1c pathway and suggests the potential therapeutic value of miR-21 for both disorders [50].

Early Detection and Screening in NASH-Related HCC

The need for a surveillance programme in patients at risk has become imperative in current times for the early detection of HCC. The screening is based on the application of a sensitive test in a repeated way and in an at-risk population, in this case, patients with cirrhosis. HCC is a disease with curative treatments increasingly available for early lesions, such as hepatic resection, liver transplantation and percutaneous ablative treatments. In addition, it can be detected by a single exam that is effective, non-invasive and low cost: ultrasonography (US) of abdomen. The primary objective of the surveillance programme is to reduce mortality or survival analysis. In terms of scientific evidence, there are three randomized controlled trials on HCC surveillance [51–53], and the benefits of regular surveillance schemes with US were analysed, showing that even with a certain heterogeneity of methodology, stages and aetiologies and with verifying the results in the same way, the increase in saved lives remained [54–62]. Each of the recent HCC guidelines of liver cancer societies in Europe, the United States and Asia, such as EASL, AASLD 2018 and APASL 2017, shows particularities. There are recommendations for HCC surveillance with evidence scores and recommendations ranging from moderate to strong. These societies agree with each other and strongly recommend HCC surveillance in at-risk populations and a surveillance time of 6 month intervals through US [63–65]. The European Liver Study Society encourages vigilance and strongly recommends such issues as the importance of implementing surveillance programmes for HCC, criteria for HCC risk populations, particularities about the population with NAFLD,

use of expert US, research of new biomarkers and cases of patients on transplant lists. Among the six strong recommendations, we emphasize that patients at high risk for HCC (cirrhotic) should participate in the surveillance programme, but the role of patients with NAFLD but without cirrhosis is still unknown. It is estimated that half of the cases with NASH and CHC occur in non-cirrhotic patients [66]. However, the incidence of CHC in these patients with non-advanced fibrosis is expected to be low to warrant universal vigilance, given the high prevalence of NAFLD in the general population. They point out that risky patients in the future can be identified and categorized as those that should and should not be monitored for HCC. Remember that the obesity of these patients is another challenge for US. Other radiological methods, such as CT or MRI, are available, but in this case, the surveillance programme would not be cost-effective. These European experts stress that the surveillance programme is vital and necessary as a medical intervention because it is cost-effective and promotes an increase in life expectancy of 3 months, costing less than approximately $50,000 per year of life saved [67]. In addition, the EASL demonstrates that in a scenario of CHC incidence greater than or equal to 1.5% per year, it is cost-effective regardless of the aetiology or the use of US and is sufficiently sensitive for tumour detection every 6 months [68, 69]. It is interesting to highlight some factors related to high risk of severe fibrosis or cirrhosis and occurrence of HCC, such as T2DM, advanced age and alcohol consumption. In addition, simple laboratory scores help identify patients with a higher risk of severe fibrosis and guarantee a greater depth of evaluation. Of particular note are the studies of genetic factors of PNPLA variants of rs738409 associated with the development of CHC in obese individuals [52, 53]. In addition, in the particular case of NAFLD, they recommend that patients with MtS or NASH identified with severe fibrosis or cirrhosis by histology or elastography should perform vigilance. EASL proposes an elaborate and compact investigation algorithm based on the size of the lesion detected by ultrasound from 1 (a) cm, in such a way as to minimize false positive results and additional costs. Such an algorithm design has been consistently validated.

Some years ago, our group published a series of NAFLD-related HCCs that exemplifies the importance of the discovery of new biomarkers for non-cirrhotic NAFLD in this context, because evaluating the applicability of the Barcelona Clinical Liver Cancer (BCLC) staging system in 42 cases of HCC secondary to NAFLD or cryptogenic cirrhosis (CC) only 52% of patients had real curative treatment according to BCLC. Additionally, HCC was diagnosed in a screening programme in 55% of the 42 patients (there was 1 non-cirrhotic patient), while patients with HCC diagnosed outside of a surveillance programme (n = 19) were mostly not candidates for curative therapy (73%) [18].

On the other hand, the American Society of Liver Studies (AASLD 2018) currently recommends the HCC surveillance programme in cirrhotic patients justified by the increased survival of these patients demonstrated by the same work by Zhang et al. [52]. In this new American guideline, it can be seen that the AASLD does not manifest itself on surveillance in populations with NAFLD, even with a growing incidence in that country. In addition, a surveillance method allow for

the option of US with or without the concomitant use of alpha-fetoprotein (AFP). The AASLD does not comment on the existing cost-effective works of the programme, which could justify its recommendations. Regarding the last APASL 2017 guideline, this Japanese society with the world's largest HCC surveillance programme favours all cirrhotic viral aetiologies even after treatment with antiviral drugs in cases of HCV. The recommendation of the programme is based on US examination and serum AFP, which is supported by moderate but scant evidence. Interestingly, HCC surveillance in East Asia recommends simultaneous use of US and AFP (but from a serum level above 200 IU), arguing that in these values, there is an increase in true positive results, resulting in less "recall" and greater efficiency of the programme.

In other words, there are currently no guidelines for the screening and early detection of NAFLD-related HCC in the absence of cirrhosis. However, there is an increasing number of HCC cases without cirrhosis. Thus, there is a strong interest in identifying biomarkers to detect early detection of HCC risk related to NAFLD progression.

In recent years, our group has been studying early detection of HCC in animal models. Because the early detection of focal hepatic lesions using US scanning is challenging and this challenge becomes even greater in the presence of diffuse parenchymal disease, we demonstrated the early detection of hepatocellular lesions in an experimental rat model of NASH with elastography and contrast-enhanced ultrasonography (CEUS). Both techniques allowed for the correct diagnosis of well-differentiated to moderately-differentiated HCC with good accuracy in an experimental rat model of NASH [70]. More recently, we demonstrated that 18F-FDG PET/CT was able to non-invasively evaluate the development of HCC in an experimental model of NAFLD. From the standardization of PET/CT in this model, it is possible to use this tool in future studies to monitor the progression of HCC non-invasively in vivo [71].

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Chapter 12
Psychological Biomarker Profile in NAFLD/NASH with Advanced Fibrosis

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Introduction

NAFLD appears as one of the main causes of chronic hepatic pathology, morbidity and mortality worldwide (mainly linked to NASH with significant fibrosis), with global prevalence estimated at around 25% [1–3]. Although this alone is alarming, its prevalence is foreseen to grow in coming decades, affecting both children and adults [4, 5]. In fact, it was recently estimated that in the next 10 years, there will be a 178% increase in cases of death related to NASH [6], so its consideration as a new twenty-first century pandemic does not seem exaggerated [7]. Many studies have been done on the influence of biochemical markers or molecular mechanisms on the course or evolution of NAFLD, however, little is known about psychological risk or protection factors that could help predict or shape the development of the disease.

In any case, the historical context in which the subject of this chapter is framed should be recalled. Until the 80s, after the proposal of the biopsychosocial model by Engel [8], the contributions of psychological, behavioral and social factors to the appearance of disease or its treatment had not been considered. However, from then on, health did not depend exclusively on biological factors, but was also directly influenced by interpretations, emotions and responses to demands from the setting. This new conception goes on to confer to the human being an active and responsible role in managing the factors that interfere with their health and therefore, disease [9].

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For an idea of how this paradigm is applied to NAFLD, we might reflect on the following example. It is quite understandable that a person with a long history of liver pathology, which advances inexorably toward cirrhosis, with the social stigma (even today, the word "cirrhosis" appears socially linked to alcoholism), develops depression. However, based on the work by Engel, we might go on to wonder if depression or some personality traits tending to depression associated with a certain lifestyle could facilitate the onset of NAFLD. Or whether it could negatively affect therapeutic adherence once the disease has been diagnosed, thereby impeding the person's stabilization in stages of light-to-moderate severity.

Throughout this chapter, special attention will be given to Type 2 diabetes mellitus (T2DM) and obesity as both pathologies are closely connected to the incidence of NAFLD. There is a consensus affirming that the exponential growth observed in the incidence of cases diagnosed with NAFLD worldwide, especially in westernized countries [10], goes hand in hand with the growing epidemic of T2DM and obesity [3, 11]. Epidemiological data show that NAFLD, NASH, liver cirrhosis and hepatocellular carcinoma are continually growing among persons with T2DM [12], placing the prevalence of NAFLD at 50–69% of cases [13, 14]. In addition, the higher the body mass index is, the higher the probability of a NAFLD diagnosis [15]. Its prevalence increases drastically up to 65% in persons with Grade I or II obesity, while in morbid obesity it is as high as 85% [16]. This figure could even surpass 90% in persons with morbid obesity subjected to bariatric surgery [17].

There is evidence that the presence of obesity and T2DM promote progression of NAFLD to its final stages that is advanced fibrosis and cirrhosis triggered by NASH [18, 19]. This, combined with a high likelihood of their coinciding with presence of liver disease, makes these two pathologies the two main risk factors associated with NAFLD [20]. Along this line we will approach psychological biomarkers that make up the NAFLD biopsychosocial profile, specifically concentrating on the following key questions: (1) NAFLD's impact on the patients' quality of life and mental health, (2) the influence of psychological variables such as coping strategies, perceived social support and perceived self-efficacy, and (3) treatment of the disease by a multidisciplinary approach.

Psychosocial Repercussions of NAFLD

Quality of Life

To understand how NAFLD affects the patients' physical and mental condition, first the disease's consequences must be considered. Concerning its clinical impact, the close relationship between cirrhosis in NASH and development of hepatocarcinoma is notorious [21]. There is growing evidence that non-cirrhotic NAFLD patients are also especially vulnerable to this type of cancer [22]. This, along with its high prevalence, makes this disease one of the main causes of liver transplantation in the

world [23], with high expectations for its leading the list in a very short time [24]. Its mortality has also increased in recent years, with advanced fibrosis as its main predictor [25, 26]. Considering that around 41% of NASH patients experience progression of fibrosis [3], advanced fibrosis would be another factor to focus attention on in this chapter.

There is broad scientific evidence confirming the negative impact of NAFLD on quality of life [27–29]. To begin with, it is notably lower in persons with NAFLD than in the general population [28], as well as in patients diagnosed with hepatitis B (HBV) or C (HCV) [30], or individuals with alcoholic liver disease, autoimmune hepatitis or cholestatic liver disease [31]. This decline in quality of life is mainly based on physical health, with no significant differences in mental functioning [27, 29, 32]. In fact, 66% of patients refer to the disease as interfering with their ability to perform daily activities [33].

Worsening of the physical aspects of quality of life may be explained largely by NAFLD symptomatology. In the first place, fatigue, often considered the most important problem for persons with NAFLD, leads to alterations of normal physical functioning [27]. Fatigue is not clearly related to severity of liver disease, but to other symptoms weakening the patient. One of them is daytime somnolence, which affects around 30% of NAFLD cases [33]. Another one is autonomic dysfunction, which involves such symptoms as vasovagal syncope or postural dizziness, and is present in both early and advanced stages of the liver disease [34].

In addition to abovementioned symptoms, other manifestations associated with NAFLD can be considered significant predictors of patients' loss of functionality, such as cognitive dysfunction [35], which leads to alterations in psychomotricity or loss of memory and concentration. Eighty-five percent of patients have slight or moderate cognitive impairment, figures similar to individuals with primary biliary cirrhosis, which is characterized by the greatest number of cognitive symptoms among liver diseases [36]. The cognitive impairment is observed both in early and late NAFLD stages, which discards its relationship with hepatic encephalopathy [37].

Moreover, the presence and severity of liver fibrosis is a determining factor in the diagnosis and prognosis of NAFLD patients, and significantly influences the impact of the disease on patients' wellbeing. Thus, an inversely proportional relationship can be observed between NAFLD severity and the physical aspects of quality of life: NASH and advanced fibrosis patients mention worse physical functionality than those with NAFLD alone. At all levels of severity, cirrhotic patients suffer from the worst quality of life [28, 31, 38].

Other determinants of quality of life in NAFLD patients may be their sociodemographic characteristics or presence of T2DM and obesity. However, the findings on the influence of respective characteristics on quality of life are inconsistent due to the selectivity of different samples [39]. Concerning sociodemographic factors in NAFLD, in some studies advanced age was related to a generalized decline in quality of life, especially in physical functioning [31, 38], whereas others did not find any correlation between the two variables [40], or they even showed an improve-

ment in emotional functioning in older patients compared to younger ones [41]. With respect to gender, empirical findings show more consistency; women with NAFLD report worse physical and mental quality of life [31, 42]. Lower formal education and socio-economic level are also significantly associated with lower mental quality of life [38].

The empirical inconsistencies become even more evident regarding the influence of T2DM and obesity. Diabetics with NAFLD show a higher likelihood of a lower physical and mental quality of life [38]. Other studies point in the same direction, confirming, however, only impaired physical functionality [40, 41]. Contrary to these findings, others did not find any significant relationship between the presence of T2DM and quality of life in liver patients [28, 30, 42].

There is some evidence with respect to comorbid obesity on the association between morbid obesity and worse physical quality of life in NAFLD [38]. In another study obese patients compared to those with normal weight reported more fatigue, less activity and more systemic symptoms [41]. However, several other studies did not show any relationship between patients' body mass index and their quality of life [28, 40, 42]. All abovementioned studies, however, did have one point in common: comorbid obesity was not associated with significant impairment in the mental component of quality of life. One possible interpretation attributes the unaffected mental quality of life in these patients to a lower cognitive and emotional involvement in issues concerning their weight. Consequently, they tend to consider the treatment received as focused exclusively on their liver disease and not on their obesity [43].

Mental Health

In research on mental health associated with NAFLD/NASH, depression and anxiety disorders are the most widely studied [44]. The prevalence of both disorders, especially that of depression, is higher in NAFLD patients than in the general population [45]. In keeping with this fact, chronic liver diseases are associated in general with anxiety and, especially, mood alterations, particularly depressive disorders [46]. The higher prevalence of depressive disorders in liver diseases is associated with a higher frequency of attempted suicide [47].

In a study with 567 NAFLD patients, the prevalence of subclinical and clinical depression was 53% and 14%, respectively, and the prevalence of subclinical and clinical anxiety 45% and 25%, respectively [48]. The clinical relevance of these data lies in the negative effects of these emotional disorders on the course and development of a disease. Thus, they increase the intensity and frequency of physical symptoms, produce functional alterations and reduce adherence to treatment thereby worsening quality of life [49, 50]. Another study found even higher rates of depression in NAFLD patients (27.2%), which highly surpassed prevalence rates in HBV (3.7%) and the general population (2–5%). Independent factors predicting

the development of a depressive episode in NAFLD were high blood pressure, lung disease, smoking and female sex of European descent [45].

Comorbid depressive disorder in NAFLD has negative consequences not only for quality of life but also a higher likelihood of progression towards more advanced stages of the liver disease [45, 48]. Furthermore, a close association between the severity of depression and the likelihood of severe hepatocellular ballooning as well as advanced fibrosis has also been observed [51]. This coincides with another study, which concluded that the link between resistance to insulin and depression and anxiety, as well as the inflammatory states which these emotional disorders generate, are determining factors in the progression of simple steatosis to NASH [52]. This line of reasoning is supported by the fact that major depressive disorder and generalized anxiety disorder become more prevalent in patients diagnosed with NASH than in individuals with mild liver damage. Specifically, presence of generalized anxiety disorder is related to more severe lobular inflammation and fibrosis than what is observed in patients without respective disorder.

Additionally, the prevalence of anxiety and depression among patients with T2DM [53] and obesity [54], which are the main comorbidities of NAFLD, is very high. Factors such as oxidative stress, weight gain as a side effect of medication, metabolic alterations, inactivity or neuroinflammation could contribute to the relationship between metabolic pathology and mental disorder [55].

Design and implementation of effective psychotherapeutic programs in NAFLD presupposes knowledge of abovementioned facts on its relationship with mental health. That is, to understand on the one hand, to what extent emotional disorders predispose to NAFLD, and on the other, the likelihood in which anxiety and/or depression develop as consequences of the liver disease.

Variables of Potential Interest in NAFLD

Coping Strategies

When people feel that internal or external demands exceed their own resources, they make an effort to confront them, using coping strategies which can be qualified as adaptive or maladaptive, depending on their results after they are put into practice [56]. Adaptive strategies, such as the search for information or support, are usually able to reduce stress and facilitate high quality of life and wellbeing in the long run. Maladaptive strategies, although they may diminish stress in the short term, have consequences which are harmful to the person's health [57]. During the course of a liver disease, the coping strategies used may be maladaptive. After diagnosis, the reaction is often anger. Individuals may also deny the existence of the pathology or give up and consequently do nothing to recover. Finally, patients may decide to take substances as a way of coping with the disease, whether drinking, smoking or unprescribed medication [58].

The negative effect of maladaptive coping strategies has previously been described for heart transplant [59], chronic pain [60], irritable bowel syndrome [61], and others. They have not been studied in NAFLD, although there are reasons justifying their importance. Nevertheless, coping strategies have been explored in chronic liver pathology. For example, in individuals with HCV a positive association has been found between maladaptive coping and decline in quality of life [58], worse perception of health [62] and longer time to diagnosis [63]. The relevance of coping in T2DM and obesity has also been studied. In T2DM this factor is a good predictor of therapeutic adherence, and can anticipate whether the course of the disease will be more or less favorable [64]. The use of coping strategies concentrating exclusively on emotion is associated with a higher likelihood of developing anxiety/depression symptoms [65]. With respect to obesity, there is strong evidence for the influence of coping on patient's mental health and physical functioning [66]. Obese individuals are usually exposed to social stigmatization, and this negatively influences their body image and self-esteem, associated in turn with alterations in mental health through anxiety/depression symptoms. Adaptive strategies for coping with this experience would be emotional expression, search for social support, confrontation or problem-solving efforts. However, maladaptive strategies associated more with anxiety symptomatology, such as self-criticism or avoidance, are common in these situations [67].

It seems reasonable to argue that NAFLD patients will use coping strategies similar to those found in T2DM and obesity, as these are their main comorbidities. This tendency might be even stronger in more advanced cases of the liver disease, where the prevalence of T2DM and obesity is greater.

Social Support

The concept of social support can be defined as perception of instrumental, emotional or economic assistance provided in the individual's most immediate environment. This usually consists of the spouse, family and friends, if the person possesses those bonds. Satisfactory support from this social network may lead to positive consequences for health, such as significant improvement in psychological morbidity or recovery from chronic pathologies [68]. There is no clear consensus on the mechanisms by which social support has such positive effects on the individual. One might argue that it acts as a modulating agent on health and quality of life, facilitating the coping with daily stressful situations [69].

Social support in chronic illness has been found to be associated with high self-esteem as well as significant reduction in the frequency and intensity of concomitant depressive symptoms [70]. With regard to our research question, there are no published data on perceived social support in NAFLD. However, this concept has been studied in other chronic liver diseases. Thus, perceiving satisfactory social support is associated with better mental health and quality of life in HBV patients [71]. Similarly, low levels of perceived social support in HCV patients are significantly

related to dysfunctional mood, anxiety and depressive symptomatology, as well as low subjective psychological wellbeing. At the same time, patients with low social support are prone to report more and stronger physical symptoms related to mobility and functional capacity [72]. Against this backdrop it is reasonable to argue that social support presumably also has important implications for mental and physical health in NAFLD.

The relationship of perceived social support to T2DM and obesity throws further light on this subject. In the first case, members of the social network significantly influence how it is managed and coped with. Adherence to a diet is higher in diabetics who perceive some type of help, whether emotional or instrumental, from persons belonging to their immediate social context [73]. The benefits of counting on strong social support in individuals with T2DM are considerable, including a tendency to show a better clinical outcome, less frequent and less severe concomitant anxiety and depressive symptomatology, and adapting better to activities in daily life [74].

In obesity, the perception of social support is one of the main predictors of subjective wellbeing [75]. Positive evaluation of social support buffers the strong deficit which is generally observed in quality of life related to physical functioning of obese individuals [76]. Social support is also an element to be kept in mind in preventing obesity, due to its relevance as a protective factor against intergenerational transmission of this illness [77].

Self-Efficacy

Perceived self-efficacy, a term coined by Bandura in his social cognitive theory [78], refers to personal beliefs about one's capacity for self-regulation and for taking action to manage and cope with a certain situation. Strong perceived self-efficacy promotes decision-making, a high sense of optimism and strong commitment to goals set. Thus, persons with high levels of perceived self-efficacy usually become involved in more challenging personal goals, investing more effort and tolerating more adaptively obstacles and difficulties [79].

The self-efficacy concept has been studied in different salutogenic behaviors, some of which are fundamental to the study of NAFLD, such as exercising, losing weight, capacity for recovering from health problems or coping with chronic diseases [80]. The positive effect that high perceived self-efficacy has on these behaviors, which lead to greater patient functional capacity and wellbeing, and in general better quality of life, has been demonstrated [81]. People with high self-efficacy also are more likely to take action for disease prevention and tend to be more optimistic about the idea of the treatment proposed culminating successfully, so it is easily inferred that therapeutic adherence is significantly greater in such cases [82].

Perceived self-efficacy has been studied in various pathologies, such as cancer [83], chronic obstructive pulmonary disease, cardiovascular disease [84] and chronic pain [85], and its importance for planning successful interventions, including modifying

the lifestyle, has been confirmed [86]. Hardly any studies have been performed on this subject in chronic liver diseases. Even so, there is evidence of a close association between the presence of anxious-depressive symptoms and low self-efficacy in these patients, which would imply a lower quality of life [58].

Study of self-efficacy in NAFLD is of special relevance with regard to its treatment, as it enables adjustment to intervention to be measured based on the individual perception of his/her disability and competence for participating in the behavioral change [87]. It is common to find lower self-efficacy among persons diagnosed with NAFLD than in other liver diseases. Particularly, a significant lack of confidence in the ability to do exercise is observed, which may partly explain the low adherence they usually show in interventions based on lifestyle changes through physical exercise. Fear of falling, a frequent emotion among NAFLD patients, could be behind this phenomenon [88].

Among Type 2 diabetics, perceived self-efficacy also has a determining role in the choice of coping strategies and self care [89]. Thus, high perceived self-efficacy is associated with adequate self care, fewer depressive symptoms and better adherence to treatment. Specifically, it is very likely that patients will carry out their diet and physical exercise as planned by their doctor [90].

With respect to obesity, several studies [91–93] have established that a primary goal of intervention for weight loss must be to increase perceived self-efficacy of their ability to carry out healthy behavior enabling them to control their weight. Another study [94] goes beyond this finding, concluding that those patients who already have high self-efficacy before intervention tend to lose less weight compared to those whose perceived self-efficacy increases during treatment. This may be explained by the fact that high self-efficacy before the intervention is indicative of excess self-confidence, thereby underestimating the difficulties entailed in losing weight. Therefore, it is fundamental to consider the pre-treatment evaluation of patients' self-efficacy and its inclusion in therapies for weight loss a primary objective of NAFLD intervention.

NAFLD Treatment: A Multidisciplinary Approach

Changes in Lifestyle

It has become indispensable to implement an effective NAFLD/NASH treatment program for prevention and lowering the likelihood of the disease progressing to its most advanced stages. No medication has yet been approved for treatment of NAFLD. To date, the most effective measure involves weight loss through changes in lifestyle [95].

The components determining NAFLD activity (steatosis, ballooning and lobular inflammation) evolve positively with weight loss [96, 97]. Specifically, loss of at least 10% of body weight is necessary to achieve significant improvements in portal inflammation or advanced fibrosis, histological parameters typical of the last stages

of the liver disease [98]. Weight loss is also associated with lowering abdominal obesity [99, 100] and risk of developing T2DM [101], as well as improvements in quality of life, especially in patients with NASH without T2DM or advanced fibrosis [42]. In patients with NASH and fibrosis, an intensive treatment for a successful change of lifestyle is vital because of its potential somatic complications [102]. In fact, one study found 64% resolution of hepatic fibrosis in patients who had received an intensive intervention with nutritionist support, compared to 20% in patients who had only received general recommendations to lose weight [103]. The change in lifestyle must include diet, physical activity and exercise [104]. It is recommended to begin with restrictive diets, avoiding saturated fats, carbohydrates and sugar-added beverages [105]. A Mediterranean diet, in turn, is established as an effective strategy in NAFLD, as its relationship with significant reductions in fatty liver disease and transaminases have been proven [106–108]. Furthermore, as a measure against low physical activity and sedentariness which usually predominate among these patients, it is recommended that their usual rhythm of aerobic activity be increased by walking for at least 60 min a day, 5 days a week. This pattern, kept up for 3 months has positive repercussions, lowering alanine and aspartate aminotransferase concentrations by half [109]. Therefore, high levels of physical activity and regular exercise protect against worsening NAFLD symptoms [44] and are associated with improvements in hepatic steatosis, inflammation and serum liver enzyme levels [97, 110].

Adherence to Therapy

Lack of adherence is the main problem in weight loss interventions for NAFLD patients: a large proportion of patients (over 50%) are unable to lose the necessary weight or regain it in a short time [54, 97]. This can be explained by the patients' lack of health awareness not thinking of themselves as being ill. The NAFLD diagnosis is not associated with an adequate increase in health awareness in the short or long term. Consequently, it does not lead to more use of health services by the patient, who does not interpret the disease as a health challenge, possibly until it advances to its last stages [111]. Therefore, it is fundamental to make patients understand their illness including risks and complications at an early stage, so that they believe in the effectiveness of medical treatment and perceive their ability to change the course of the disease by changing their future lifestyle. This may be particularly challenging, when their previous lifestyle was very different and greatly unhealthy [112, 113].

Patients' emotional state is also a relevant element in this analysis, as depression and disease-related emotional stress are associated with low self-efficacy, which in turn has negative consequences for adherence to therapy and lifestyle changes [51, 114].

Another key question for understanding problems with adherence to therapy in NAFLD patients is the lack of willingness to change resulting in little motivation to follow the health professionals' guidelines, in particular concerning physical

activity. This is particularly alarming considering that physical and mental symptoms following severe liver damage do not strengthen willingness to change, quite the opposite [111, 115]. There are some strategies the health professional can use for improving patients' motivation to change their lifestyle: (1) a collaborative style which attracts, motivates and commits the patient, (2) evaluate, along with the patient, the advantages and disadvantages of the treatment, (3) promote self-efficacy by designing individualized plans, focusing feasibility, (4) analyze with the patient all variables that maintain the unhealthy lifestyle, (5) explain the treatment in detail, and (6) increase the patient's awareness of the negative impact of the social stigmatization of obesity [116, 117].

To further strengthen adherence, it is also recommended that health professionals encourage a clearly structured diet, which limits the patient's choices, so that the likelihood of eating undesirable foods and making mistakes in calculating the daily amount of calories is reduced [118]. The proposed diet may include planned meals, menus and recipes [119]. In contrast to the recommendations concerning diets, some studies have noted that adherence to physical exercises increase when they are less structured. From this viewpoint, the likelihood of successfully implementing a plan for physical activity would be better if carried out individualized at home instead of taking part in a supervised workout program alone or in group sessions in a gym [120, 121].

Cognitive-Behavioral Treatment

Interventions in NAFLD must provide patients with enough tools to achieve the therapeutic goals and include them in their daily habits, maintaining the results in the long term. To achieve this, the traditional recommendations for changes in lifestyle must be replaced, especially in individuals at risk of advanced liver disease, by implementing a cognitive-behavioral treatment (CBT) [122, 123].

CBT attempts to modify maladaptive patterns of thinking and behavior to achieve improvement in an individual's mood and psychosocial functioning [54]. In this sense, CBT techniques recommended for intervention in NAFLD are: (1) controlling stimuli, which consists of modifying the context, eliminating signals leading to the problem behavior and increasing those leading to the desired response. For example, by not allowing forbidden foods to enter their home and placing those recommended in an accessible place [118], (2) setting realistic personal goals and commitments, for example, "I'll only eat when I'm at the table" or "I am going to lose half a kilo a week" [118], (3) writing down everything eaten, physical activity and weight using self-report questionnaires [124], (4) reinforcing alternative behaviors, where the patients, for example, learn to identify signals that can lead them to eat without being hungry, replacing this response with other options, such as showering, exercising or using relaxation or distraction techniques [86], (5) problem-solving strategies to approach obstacles to weight loss or maintenance, by planning a series

of steps to be able to cope with them successfully [86], and (6) cognitive restructuring which promotes development of a more adaptive style of thinking, including interventions for cognitive bias and unrealistic expectations related to weight and weight loss, which are significantly linked to quitting therapy [118, 125].

Inclusion of CBT in NAFLD interventions is associated with positive changes in patients' health. Normalization of liver enzymes, improvements in sensitivity to insulin and greater weight loss in the short and long-term should be emphasized [126]. For its assured implementation, in addition to a well-defined program with the techniques described above, a multidisciplinary team of medical and nursing staff, psychologists, nutritionists and experts in physical exercise is recommended, which assists the patients during treatment sessions and can flexibly react to their needs [86, 127]. It has been found that multidisciplinary interventions are associated with better clinical results and higher patient satisfaction than standard care [128].

Unfortunately, lack of resources often impedes multidisciplinary approaches, so it is essential to promote NAFLD training programs for physicians. These include pragmatic interventions for promoting lifestyle behavior changes in problematic patients [112, 113, 129, 130]. The clinical relevance of NAFLD often is undererstimated and knowledge of the disease is inadequate, especially among physicians who are not hepatologists [131–133]. Add to this the urgent need for public information and education programs on NAFLD [129, 134] by increasing knowledge of the disease through the communications media, and by strengthening physical activity in schools and providing incentives to use parks or bicycle lanes for exercise; warning about junk food, ultra-processed and sugar-added foods, especially regulating advertising directed at children, and promoting access for the lower socio-economic population groups to healthy foods [135].

In this chapter we have reviewed the psychosocial biomarkers associated with NAFLD. This is a subject that should be studied in greater depth in the coming years, as to date there are only few and sometimes contradictory studies. In spite of this, we have analyzed the quality of life of persons with NAFLD, and how it is affected by such factors as fatigue, anxiety, depression and cognitive dysfunction. We have also examined further relevant variables, such as coping strategies, perceived social support and perceived self-efficacy, which are understudied in NAFLD. However, their relevance for subjective wellbeing in obesity and T2DM, which are the main comorbidities of NAFLD, makes it highly likely that they also play an important part in mental health and quality of life in NAFLD. Finally, we approached matters related to treatment of NAFLD and came to the conclusion that in hospitals multidisciplinary teams are necessary, where psychologists, nutritionists and other health professionals cooperate with doctors and nurses in the design and implementation of medical therapy, cognitive-behavioral intervention programs and awareness campaigns.

The significant impact of advanced liver disease on patients' lifes as well as its massive economic and societal implications demand every effort for the optimization of prevention as well as treatment programs.

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Chapter 13
Integrative Proposal for the Use of Biomarkers in Clinical Practice Management of NAFLD/NASH

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is reaching epidemic proportions and is currently considered the most common liver disease worldwide [1, 2]. The estimated global prevalence has been estimated to be 24–25% of the general population and an exponential increase in NAFLD-related burden is expected to occur in the upcoming years based in recent epidemiological modeling studies [3]. The worldwide continuously increasing rates of adult overweight and obesity [4], type 2 diabetes mellitus (T2DM) [5] as well as the high prevalence of lack of physical activity and increasing sedentary behavior [6] are the main factors driving the global increase in NAFLD prevalence, which will likely deter a related increase in liver-related morbidity and mortality in the near future [2, 7].

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Identification of NAFLD and discrimination of the different subgroups of patients, grouped under this umbrella term are key issues in the field of liver diseases [8]. These different subgroups are heterogenous and include subjects with different predominance or hierarchy of pathogenetic mechanisms and distinct natural history as well as individuals at different stages of the disease, which have different liver and non-liver related outcomes [9–11]. While liver biopsy may still be used to diagnose and stage the disease [12], refining noninvasive tools (NITs) to appropriately asses the presence of NAFLD and its severity or degree of progression has been a key goal of the field in the last decade [13, 14].

In the previous chapters, current concepts regarding the development and performance of biomarkers allowing to diagnose NAFLD and discriminate among patient subgroups (i.e. patients with isolated steatosis, patients with nonalcoholic steatohepatitis [NASH] and patients with advanced fibrosis or cirrhosis) have been extensively reviewed. In this chapter and, based in the aforementioned reviews, we aim to propose rational use of selected biomarkers when evaluating patients with NAFLD. A brief summary of the information regarding available tools to assess NAFLD presence and severity is provided below followed by a proposal of theier clinical use at present time.

NAFLD Diagnosis

The presence of NAFLD is usually diagnosed based on evidence of steatosis in imaging studies or histology along with absence of significant alcohol consumption and no coexisting causes of liver disease. Since most patients with NAFLD are asymptomatic [15, 16], diagnosis is frequently incidental. Thus, an increased liver echogenicity found in an abdominal ultrasound done for other reasons is the most clinically common scenario leading to NAFLD diagnosis. Also, if abnormal liver tests are present, that generally triggers the diagnostic process and lead to establish the presence of NAFLD after other etiologies of liver disease are excluded. Active screening of NAFLD is not generally pursued but in the context of epidemiological or clinical research. That said, active screening for NAFLD among higher-risk individuals, such as those with T2DM [17, 18] and metabolic syndrome (MetS) [19, 20], might be warranted and is recommended for some major scientific societies such as the *European Association for the Study of the Liver* (EASL) [21], *The Asian Pacific Association for the Study of the Liver* [APASL] [22] and the *British National Institute for Health and Care excellence* (NICE) [23].

Clinically available tools to detect NAFLD include several equations (i.e. Fatty Liver Index (FLI), the Kotronen score, the Lipid Accumulation Product (LAP), the Korean study score, the Hepatic Steatosis Index (HSI), and the SteatoTest [see Chap. 6 for details]), which might help to suspect NAFLD and imaging techniques with abdominal ultrasound (US) being the preferred technique due to its easy availability, its non-invasive nature, low cost, rapidity and radiation-free-features. Of note, most of the above-mentioned equations have been validated mainly for

epidemiological studies and its utility in clinical practice is limited. Although these biomarker panels could be useful in screening of NAFLD in at-risk populations, at present time, abdominal US represents the more pragmatic first-line investigation. Thus, abdominal US should be offered in those patients with supected NAFLD (i.e. patients with overweight, obesity and/or features of MetS and/or altered liver tests). The pooled sensitivities and specificities of abdominal US to distinguish moderate-to severe fatty liver from the absence of steatosis, have been estimated as being 84.8% and 93.6%, respectively [24]. Limitations of abdominal ultrasound in diagnosing of NAFLD include its low sensitivity for detection of steatosis less than 20–30% and operator dependence (see Chap. 7 for details).

Other techniques such as computed tomography and magnetic resonance imaging (MRI) techniques including magnetic resonance spectroscopy (MRS), multi-echo MRI [25] and proton density fat fraction (PDFF) [26] (reviewed in Chap. 8) are at present time used mainly in research settings but may have a diagnostic role in the clinic in cases where fat is not homogenously distributed with complex zonal or segmental steatosis.

One important advantage of MRI-based techniques is their ability to more precisely with MRI-PDFF being able to estimate hepatic PDFF across the entire liver. For that reason, MRI-PDFF is considered as a very good imaging tool for NAFLD diagnosis [13, 26]. However, the precise estimation of the amount of liver fat is yet of uncertain clinical significance and therefore, at present time, quantification of hepatic fat is mainly carried out in research settings. New and simpler techniques such as controlled attenuation parameter (CAP) are emerging as useful for quantifying severity of hepatic steatosis [27]. Although CAP is outperformed by MRI-PDFF, it may be more precise than ultrasound to detect mild steatosis in clinical practice although more studies are needed before its routine use be recommended [13, 28].

Diagnosis of NASH

NASH is an histological diagnosis defined by presence of necro-inflammatory and cell degeneration changes (neutrophil infiltrates, apoptosis, cellular ballooning) in liver biopsy [29, 30]. Patients with NASH seem to have a different natural history than no-NASH patients [11, 31]. It has been estimated that up to 30% of those patients with NASH may evolve to advanced liver fibrosis or cirrhosis over one decade [10, 11, 32]. Thus, identification of patient with NASH is important as they represent an at-risk group to develop liver outcomes. Current guidelines state that NASH should be diagnosed histologically [15, 21] but liver biopsy is a feared procedure due to its invasive nature and other drawbacks such as sampling error and cost [12]. Thus, search of noninvasive markers of NASH has been intense in the last two decades. However, in spite of intense research, currently there are no biomarkers able to effectively differentiate NASH from simple steatosis with a robust sensitivity and specificity [33]. The use of determinations of serum levels of cytokeratin (CK)-18 has been extensively studied and although in some studies have shown

good performance, its use is limited due to several factors such as lack of availability, limited sensibility and a poor definition of the cut-off to be used [13, 34]. Additional NASH biomarkers have been investigated including panels combining some inflammatory markers, lipid oxidation products and other lipid species adipocytokines and lysosomal enzymes but independent validation is lacking, and none of them are currently ready for widespread clinical use [14, 33]. The use of metabolomics (reviewed in Chap. 10) hold promise but refinement and further validation is needed [35]. A recent report showed that the use of a noninvasive lipidomic serum tests based in measurement of several triglycerides species was able to distinguish between NASH and NAFL with high accuracy [36] and could represent an easy-to-use tool to differentiate disease severity and potentially to monitor disease progression. However, further validation is needed.

Assessment of serum micro-RNAs profiles have been also suggested to be useful toi diagnose NASH [37]. Circulating levels of miR-122 and miR-34a are potentially suitable biomarkers for NAFL/NASH discrimination but need further validation in large human cohorts [38].

Lastly, it is important to stress that, at present time, no imaging modality can reliably discriminate NASH from simple steatosis, although some refinement in ultrasound and MRI-based modalities have shown advances in this regard [13, 39, 40].

In conclusion, in absence of a good biomarker to discriminate between isolated balnd steatosis and NASH, the use of clinical features can indeed be of help to identify patients with higher chances of having NASH. Features to be considered in this regard include the following: patients with more than 50 years, persistently elevated serum aminotransferases or presence of severe obesity, T2DM and/or MetS. If judged clinically necessary, NASH diagnosis should be confirmed by a liver biopsy [15, 29].

Diagnosis of Fibrotic NAFLD/NASH

Assessment of hepatic fibrosis in subjects with NAFLD can be done with a wide variety of NITs (for a comprehensive review see [13, 41] and Chap. 6 of this book). Among them, the non-proprietary FIB-4 and NAFLD fibrosis score are recommended by current guidelines as useful tests for initial evaluation of patients with NAFLD [15]. FIB-4 and NAFLD fibrosis score are readily available and easy to calculate with good reproducibility and a high negative predictive value allowing excluding the presence of advanced liver fibrosis. Positive predictive value of NFS and FIB-4 are less accurate and results suggesting fibrosis should trigger further evaluation [13]. Other patented tools (FibroTest, Fibrometer, Hepascore, Enhanced Liver Fibrosis [ELF] score have been studied with a generally good area under the receiver operating curve (AUROC), being higher than 0.8 for most of them,

although no independent meta-analysis examining their performance has been published. Additional tools have recently published using indicators of type III collagen synthesis (i.e. PRO-C3) [42] or measurement of other biological parameters such as alpha-2 macroglobulin (A2M), hyaluronic acid (HA), and tissue metalloproteinase inhibitor 1 (TIMP1) [43] but further independent confirmation is needed. It is important to consider that most of the NITs currently available were developed and validated in non-diabetic patient cohorts and that they may underperform in T2DM patients [13]. More recently, a new non-patented scoring system (Hepamet Fibrosis Scoring System) has been developed and validated in several cohorts of various ethnic origins [44]. This new system identified patients with advanced fibrosis with greater accuracy than FIB-4 and NFS systems and may have better performance in diabetic patients.

Among the additional tools to assess the presence of liver fibrosis and its severity, Vibration controlled transient elastography (VCTE) is very accurate for diagnosing advanced fibrosis in patients with NAFLD with sensitivities of 85 and 92% and specificities of 82 and 92%, respectively [13]. Although it has some limitations (reduced performance in obese patients and existence of confounders such as cholestatic liver disease and acute hepatitis [13, 33]), VCTE is a point-of-care technique of relatively low complexity that is recommended by current guidelines for identification of those patients at low or high risk for advanced fibrosis [15]. Magnetic resonance–based elastography has been shown to be more accurate than VCTE, but is more expensive and less available and currently used mostly in research settings [45] (see Chap. 8 for details). Other techniques are being developed in recent years (i.e. Acoustic Radiation Force Imaging/Point shear wave elastography (pSWE) and 2D-shear wave elastography (2D-SWE)) (see Chap. 7 for details) and are promising. However, more validation studies are needed before their widespread use can be recommended. Among those techniques, 2D-SWE seems to have an equivalent performance to VCTE [46].

Combination strategies attempting to identify subjects with fibrotic NASH have been proposed [47]. In this line, a recent oral communication assessed the usefulness of a combination of serum levels of aspartate aminotransferase with VCTE and CAP and reported a good diagnostic accuracy of this combination for identification of patients with NAS ≥4 and ≥F2 fibrosis stage, with a pooled AUC of 0.82 [48]. Also, a stepwise approach (fibrosis assessed initially with either NFS or FIB-4, followed by VCTE) improves performance particularly in subjects with intermediate NFS and FIB-4 scores [13], thus avoiding unnecessary referrals. A recent report by Boursier et al. propose the sequential use of FIB-4 and a combined tests including VCTE and the proprietary test Fibrometer to further reduce the "gray zone" of results [49]. Thus, a multi-staged or stepwise approach may be more useful than using a single test to non-invasively diagnose fibrosis degree in patients with NAFLD [50, 51].

Diagnosis of Cirrhosis

Evidence for several studies suggest that a significant proportion of patients with NASH-associated cirrhosis remain not identified [47, 52, 53]. In fact, unless that obvious signs of cirrhosis are present (i.e. splenomegaly, morphologic alterations of the liver, collateral circulation, etc.) in imaging studies [54, 55] diagnosis might be overlooked. It should be kept in mind that liver tests correlate poorly with histological findings and normal values do not exclude significant liver disease including cirrhosis [56]. Use of serological markers (i.e. platelets) and equations (FIB-4, NFS) can be used as a first tool to rule out cirrhosis and, if abnormal, a second test is in order. VTCE is a very useful tool for detecting cirrhosis when appropriate cut-off points (higher than 12.5 kPa) are used with reported accuracy is higher than 90% in absence of confounders. Point shear-wave elastography (2D-SWE) seems also have a good performance in diagnosing cirrhosis [46] and MRE has been shown to outperform other techniques [55, 57] but with more limited access.

Use of Diagnostic Tools and Biomarkers to Assess NAFLD in the Clinic

Decision on the clinical use of the described tools and biomarkers in the clinical setting will depend on local availability, cost-efectiveness considerations and evidence-based analysis [55]. Also, biomarkers and imaging techniques must be interpreted critically, considering the setting of use (primary care or tertiary referral centre) and the clinical context of the patient under evaluation [13]. Lastly, quality criteria for each test and possible pitfalls should be taken into consideration [58].

Given that underappreciation of the prevalence and clinical spectrum of NAFLD and its assessment among non-specialists has been documented [59, 60], educational activities to increase and knowledge on NAFLD targeting those physiciants caring for patients *at-risk* are needed. In particular, the use of simple and freely available scores (FIB4/NFS) to detect liver fibrosis should be promoted as a first step of evaluation of NAFLD patients [13, 59]. In patients with T2DM and MetS, NAFLD should be actively sought followed by apropriate evaluation of the presence of NASH and liver fibrosis [21, 61]. Ideally, and based in some observational studies showing a high prevalence of advanced fibrosis and cirrhosis in T2DM [62, 63], assessment of liver stiffnes by VCTE or 2D-SWE should be the chosen technique given the potential limitations of FIB4/NFS in T2DM patients [64, 65]. Although patented test might provide a slight improvement in diagnostic accuracy over other biomarkers, their limited and cost might limit their application in most settings. Thus, at present time FIB4 and NFS are recommended by experts as part of a pragmatic first-approach when evaluating patients with NAFLD [13].

Figure 13.1 presents a suggested algorithm for patients suspected of NAFLD/NASH, which can be modified according to available resources or new evidence.

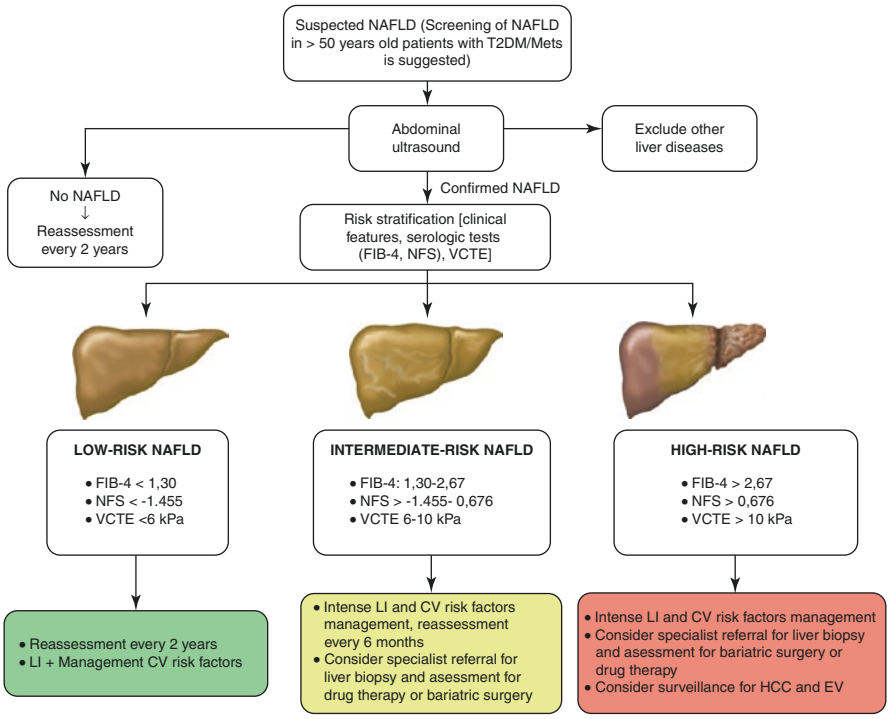

Fig. 13.1 Suggested algorithm for evaluation of non-alcoholic fatty liver disease (NAFLD) and NAFLD-related liver fibrosis using noninvasive tools (NITs). While NAFLD can be suspected based in clinical features (i.e. overweight, obesity dyslipidemia, etc.), Screening for NAFLD is advised in type 2 diabetes mellitus (T2DM) patients and patients with metabolic syndrome (MetS) older than 50 years-old. Abdominal ultrasound is the preferred method for steatosis identification although its performance for mild steatosis is not ideal. Assessment of clinical features (particularly chronic liver disease stigmata, serum liver test and platelet count) might help to identify those patients that already developed cirrhosis and may have lost fat in the liver (burned out cirrhosis). Fibrosis evaluation with noninvasive scores such as Fibrosis 4 (FIB-4) and NAFLD fibrosis score (NFS) can be calculated from laboratory variables as triaging tests. If abnormal values are found, evaluation should be followed by liver stiffness assessment with vibration controlled transient elastography (VCTE) to evaluate liver fibrosis and decision of hepatology consultation. Sequential use of FIB-4 and elastography is advised but VCTE could also be used as triaging test if available and may be indicated as a first test in T2DM patients. *LI* lifestyle interventions, *CV* cardiovascular, *HCC* hepatocellular carcinoma, *EV* esophageal varices. FIB-4 can be calculated at https://www.hepatitisc.uw.edu/page/clinical-calculators/fib-4.N NFS can be calculated at http://gihep.com/calculators/hepatology/nafld-fibrosis-score/

The cost-effectiveness of this approach await for validation but roughly represent our daily clinical practice. The first step imply triaging those patients that may remain in the setting of primary care or should be referred [59]. Those patients with NAFLD in whom NASH is unlikely and no evidence of fibrosis have been found (FIB-4 <1.3, normal VCTE values) have a very-low risk of liver events and are esentially at risk of cardiovascular outcomes [66, 67]. Therefore, interventions should be

organized at primary care level after appropriate evaluation of cardiovascular risk. Patients with suspicion of NASH and no evidence of significant fibrosis should be referred for hepatology consultation in order to exclude other causes of liver diseases and consideration of performing a liver biopsy. If a high suspicion of NASH is present or the diagnosis is confirmed, focus on intensive lifesyle interventions is indicated, ideally in the context of multidisciplinary teams at primnary or secondary level. Implementation of specific programs aiming to control excessive body weight, promote healthy dietary habits and physicial exercise with the aid of dedicated websites [68, 69] and smart phones apps is desirable in joining efforts with cardiovascular risk management programs. Re-evaluation every 4–6 months should be made and estimation of NASH resolution using online tools should be performed [70]. If after 12 months there is no evidence of improvemente consideration of drug treatment or indication of bariatric surgery could be made. Patients with NAFL/NASH and evidence of significant fibrosis or cirrhosis should be also referred for evaluation. These patients should be considered for more detailed assessment of fibrosis with MRE if the technique is available and eventually undergo liver biopsy. If significant fibrosis is present, in addition of intensive lifestyle interventions consideration should be made of implementing pharmacological treatment either with the currently available agents or in the context of clinical trials. Finally, if cirrhosis is present, screening of esophageal varices and hepatocellular carcinoma is indicated as part of general management of cirrhosis.

Conclusions

Triaging of different patient populations grouped under the term of NAFLD into defined clinical pathways is crucially important for rational use of healthcare resources. Available tools to differentiate among these populations are still imperfect but had improved significantly in recent years. Since cost-effectiveness studies to evaluate the value of different strategies for triaging patients with NAFLD as well as the screening patients at risk for the disease are lacking, current recommendations are based on available information, expert opinions and personal views that could be adapted to local scenarios.

Evaluation of liver fibrosis is mandatory in NAFLD, as advanced fibrosis identifies the subgroup of patients with impaired prognosis. In this regard, at present time, he use of non-patented and simple scores (FIB4/NFS) and, if easily accesible, VTCE are currently considered as the first steps of evaluation of patients with NAFLD aiming to define clinical management according to disease stage. It is also important to stress that, giving the underapprecciation of NAFLD among non-specialists, awareness about the disease and its evaluation with simple NITs should be promoted in both primary care setting and among physicians caring for patients with metabolic disturbances in order to capture those patients at risk of adverse outcomes. Further refining of noninvasive diagnosis will likely contribute to close the current existing gaps of information and improve our capacity to diagnose and staging NAFLD.

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