Chapter 1

Diagnosis and Characterization of Non-Alcoholic Fatty Liver Disease

Paula Iruzubieta, Marta González, Joaquín Cabezas, María Teresa Arias-Loste and Javier Crespo

Additional information is available at the end of the chapter

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Abstract

Non-alcoholic fatty liver disease (NAFLD) can develop cirrhosis and even hepatocellular carcinoma, resulting in a high liver-related morbidity and mortality, being important to know those risk factors for disease progression, among which the presence of diabetes stands out. In addition, it is a disease with multisystemic behavior, becoming an independent risk factor for cardiovascular disease and extrahepatic tumors. Hence, early diagnosis and multidisciplinary management of NAFLD are really important. In this chapter, we will expose the different diagnostic and follow-up tools available for this disease, and with them we will make an algorithm according to the recommendations and the current evidence.

Keywords: NAFLD, biomarkers, transient elastography, multisystemic disease

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) includes a wide spectrum of liver damage whose distinctive feature is the accumulation of intrahepatic fat, especially triglycerides, which cannot be attributed to secondary causes such as alcohol and certain drugs. NAFLD is nowadays considered to be the most common cause of chronic liver disease in western countries, showing a prevalence of around 30% in the general population [1]. Within NAFLD, two histological subtypes can be distinguished: (a) non-alcoholic fatty liver (NAFL), which includes patients with simple steatosis with or without mild inflammation and (b) non-alcoholic steatohepatitis (NASH), characterized by the presence of hepatic inflammation and hepatocyte injury (ballooning) with or without fibrosis [2, 3]. NAFL is a generally benign condition, and NASH is the progressive subtype that can lead to cirrhosis and hepatocellular carcinoma

(HCC) [4]. However, several studies with paired liver biopsies have demonstrated that both patients with NASH and those with NAFL have the potential to develop a progressive hepatic disease, and in this risk of progression there are some key factors such as diabetes mellitus [5, 6]. In general, patients with NAFLD have a higher long-term mortality than the general population, cardiovascular disease (CVD) being the principal cause of death, followed by different types of cancer [7–9] and liver-related complications, as well as the cardiovascular risk caused by the different factors of the metabolic syndrome, very frequent in this type of subjects; NAFLD is itself an independent risk factor for CVD [10]. Liver-related mortality is increased up to 10-fold in patients with NAFLD. In this sense, it should be emphasized that cirrhosis and HCC are the fifth most prevalent cause of mortality in the world. Therefore, given the hepatic and cardiovascular morbi-mortality generated by NAFLD, the early identification of these patients is important to provide suitable management that can lower the mortality for all causes.

2. Screening and diagnostic criteria

The mechanisms leading to the development and progression of NAFLD are not completely known, but it is widely accepted that the initial events are dependent on the development of obesity and insulin resistance (IR) [11]. For this reason, NAFLD has a strong association with the factors constituting the metabolic syndrome, the prevalence in this group of patients being considerably heightened. This relation is especially close in morbid obesity, where NAFLD is present in more than 90% of the cases, this condition taking the form of steatohepatitis in a third of the cases, while in up to 5–10% of the subjects, the liver disease has progressed to cirrhosis [12, 13]. The association between NAFLD and IR or diabetes mellitus type 2 (DM2) has also been clearly established [14]. It has been demonstrated that DM2 is associated with a greater hepatic content of triglycerides independently of the body mass index (BMI) [15, 16]. Thus, the prevalence of NAFLD in DM2 patients can reach up to 70% [11, 17, 18]. Moreover, both prediabetes (glucose intolerance and altered glucose when fasting) and DM2 are related to the severity of liver damage, the presence of steatohepatitis, fibrosis and even HCC [1, 19, 20]. Overall, 80% of the NAFLD cases present with some of the cardiovascular risk factors that constitute the metabolic syndrome (IR, obesity, dyslipidemia and arterial hypertension), and its prevalence directly increases the number of these factors that are present [21].

As a consequence of its high prevalence, especially in subjects with the abovementioned risk factors, its prognostic implications, and given that NAFLD is generally an asymptomatic disease, some authors recommend the implantation of an NAFLD-screening programme within the risk population [22, 23]. However, this topic is at present controversial given the great load on the national health systems that could be caused by these screening programmes and the lack of efficacious treatments currently available. In fact, the principal associations for the study of liver diseases (American Association for the Study of Liver Diseases (AASLD) and European Association for the Study of the Liver (EASL)) in their guidelines for clinical practice do not recommend this screening in any population [24] or they recommend it, with an A2 level of evidence, only for patients with DM2 independently of the levels of hepatic

enzymes [25]. To attempt to answer this question, valid cost-utility studies are necessary in screening programmes. There is no discussion, however, about the need to act when faced with a patient suspected of having NAFLD and not to underestimate its discovery due to the limited clinical and analytical repercussion manifested at first. In our opinion, patients with NAFLD and suspected to have advanced disease must be evaluated in specialist units for their correct characterization in case of a prompt availability of specific treatment.

2.1. Clinical and analytical manifestations

A diagnosis of NAFLD is very often reached through a casual analytical discovery during a health examination after an alteration in tests of liver function, or an alteration in hepatic morphology detected through an image study done with another objective, given that it is generally an asymptomatic disease. In the cases in which the patient reports symptoms, they are usually mild and unspecific, asthenia and abdominal problems being frequent, especially in the right hypochondria. The physical exploration may be normal or detect a soft, painless hepatomegaly, although occasionally it is difficult to evaluate as these patients very often present with central-type obesity, and in the patients with advanced fibrosis and cirrhosis, we may find signs of portal hypertension such as ascites, splenomegaly or jaundice [26].

Analytically, most of the patients present tests with normal or discretely altered liver function, with a predominance of ALT (alanine aminotransferase) compared to AST (aspartate aminotransferase). On specific occasions, a discrete elevation can be appreciated in the markers of cholestasis, especially GGT (gamma-glutamyl transpeptidase), which has been related to obesity and IR [27]. Another frequent analytical discovery is the elevation of the levels of ferritin in blood and of the transferrin saturation index without having demonstrated a corresponding increase in the deposits of hepatic iron [28]. Something similar occurs with the presence of elevated autoantibodies, which appear quite frequently in NAFLD and are considered an epiphenomenon [29].

2.2. Diagnosis of steatosis

Hepatic steatosis is defined histologically as the deposit of fat ≥5% of the hepatocytes and is classified in four grades depending on the percentage of hepatocytes with steatotic vacuoles. The normal liver (S0) contains fat in less than 5% of the hepatocytes while grade 1 steatosis (S1) corresponds to less than 33% of the steatotic hepatocytes. In grade 2 and 3 steatosis, fat is present in at least 33 or 66% of the hepatocytes, respectively.

The presence of risk factors such as DM2, metabolic syndrome and obesity with the elevation of the hepatic enzymes, especially ALT, increases the possibility of fatty liver presenting. Nevertheless, although the ALT is a useful test, it is not valid for predicting the presence of this disease, or even the risk of progression, given that it can occur with normal hepatic enzymes [30]. In fact, in patients with DM2 and normal levels of ALT, a high prevalence of NAFL and NASH has been reported [31].

In clinical practice, ultrasound scan is a first-rate image technique if NAFLD is suspected due to its wide availability, low cost and safety [32]. The sensitivity of this technique is 93%

when the steatosis is greater than 33%; however, this sensitivity decreases considerably when the steatosis affects less than 30% of the hepatocytes [33, 34]. Steatosis can also be diagnosed through computerized tomography (CT), but its cost and the patient's exposure to radiation make its systematic use in long-term follow-up unadvisable in this pathology; moreover, its sensitivity does not improve substantially if the steatosis is mild [32]. Magnetic resonance imaging (MRI), including spectroscopy, can diagnose content levels of hepatic fat >5% and it is reliable to determine changes (≥0.5%) in the grade of steatosis after weight loss. Although its use has widened in many studies, its use in clinical practice is limited by its cost and duration [35, 36].

The recently developed CAP (controlled attenuation parameter), an application of transient elastography (TE), which will be discussed later, available in the latest generation devices, enables the immediate and easy quantification of steatosis. CAP measures the degree of attenuation of the ultrasound wave transmitted through the liver, which is proportional to the amount of hepatic fat, and is less influenced by the sampling error than the liver biopsy, since it explores a liver volume approximately 100 times greater. Its values oscillate between 100 and 400 dB/m and it is possible to measure the liver stiffness used for the evaluation of fibrosis simultaneously. The studies published to date indicate that CAP is capable of diagnosing steatosis in chronic liver diseases of diverse causes even in mild stages (>10%) and has a good correlation with the degree of steatosis [37–44]. These studies show different cut-offs of CAP for the different grades of steatosis, but all of them demonstrate that the cut-offs do not differ among the different causes of liver diseases, in contrast with what happens with transient elastography [40]. In this sense, a recent meta-analysis including 2735 patients has established a series of cut-offs for the different grades of steatosis: 248 dB/m for S1, 268 dB/m for S2 and 280 dB/m for S3, with a sensitivity of 69, 77 and 88%, respectively, and a specificity of 82, 81 and 78%, respectively. In this meta-analysis, etiology, BMI and diabetes showed a significant influence on the value of the CAP, so the authors suggested the cut-offs established but subtracting 10 dB/m from the value of the CAP for the patients with NAFLD, 10 dB/m for diabetics, and subtracting/adding 4.4 dB/m for each unit of BMI above/below 25 kg/m^2 in the interval of 20–30 kg/m^2 [45].

Another image technique is magnetic resonance imaging-estimated proton density fat fraction (MRI-PDFF), which is based on chemical shift-based water fat separation methods. MRI-PDFF has shown good correlation with histology-determined steatosis grade in NAFLD patients, so it could be used for follow-up and treatment-response evaluation [46].

Lastly, within the non-invasive diagnosis of steatosis, various serological tests of biomarkers have been developed to predict the existence of hepatic fat (**Table 1**) [47–51]. However, all these biomarkers may be influenced by inflammation and fibrosis, and given that they do not provide great advantages compared to image techniques and routine analysis, their use in clinical practice is not widespread; even so, the Fatty Liver Index (FLI) that uses easily available parameters could be considered, although CAP has demonstrated better performance than this test for the diagnosis of grade 2–3 steatosis [44]. Nevertheless, FLI has been associated independently to liver-related mortality, as well as to the mortality rates due to

Indices	Formula	Cut-offs	Sensitivity (%)	Specificity (%)
Hepatic	8 × ALT/AST ratio + BMI	30 (low cut-off)	93	40
Steatosis	(+2, if DM; +2, if female)	36 (high cut-off)	45	93
Index (HSI)				
Fatty liver	exp(*n*)/1 + exp (*n*) × 100	10 (low cut-off)	95	29
Index (FLI)	(*n*) = 0.953 × ln(TG) + 0.139 × BMI + 0.718 × ln(GGT) + 0.053 × waist circumference – 15.745	60 (high cut-off)	44	91
SteatoTest	Proprietary formula (a2-macroglobulin, haptoglobin, apolipoprotein A1, GGT, bilirubin, ALT, cholesterol, triglycerides, glucose, BMI, age, gender)	0.3 (low cut-off)	90	54
		0.7 (high cut-off)	46	88
NAFLD liver Fat score	−2.89 + 1.18 × metabolic Syndrome (yes: 1, no: 0) + 0.45 × Type 2 diabetes (yes: 2, no: 0) + 0.15 × insulin + 0.04 × AST − 0.94 × AST/ALT	−0.640	86	71
Lipid accumulation product (LAP)	LAP (men) = Waist circumference – 65	20 (low cut-off)	99	16
		80 (high cut-off)	43	94
	LAP (women) = Waist circumference − 58			

Table 1. Indices for diagnosis of steatosis.

cardiovascular disease and cancer, but it seems that these associations are interfered by the risk conferred by the state of insulin resistance [52].

When steatosis is suspected in the aforementioned non-invasive methods, the liver biopsy is still the gold-standard method to conclusively diagnose NAFLD and the only one capable of distinguishing between NAFL and NASH, thus enabling the classification of the disease

according to the grade of activity (inflammation and hepatocyte injury) and the stage of fibrosis, the best predictors of the disease progression [53, 54]. The advantages and, especially, the drawbacks of the liver biopsy are dealt with later.

2.3. Initial diagnosis of NAFLD

From what has been mentioned so far, we can specify a series of characteristics that indicate a patient with NAFLD: (1) radiological evidence of steatosis or CAP >248 dB/m ± Abnormal liver blood test, (2) the presence of insulin resistance or another component of the metabolic syndrome, (3) consumption of alcohol of <30 g/d in men and <20 g/d in women and (4) exclusion from other causes of chronic liver disease (viral hepatitis, cholestatic diseases, autoimmune hepatitis, hemochromatosis, α1 antitrypsin deficiency, Wilson's disease, drug-induced liver injury and celiac disease) [24, 25]. Once the initial diagnosis of NAFLD has been made, our next step is to evaluate the stage of disease and the necessity of carrying out a liver biopsy.

3. Diagnosis of steatohepatitis

A key element in the diagnosis of NAFLD is the differentiation of NASH from NAFL and the staging of the liver fibrosis, given that patients with NASH and advanced fibrosis are those at the greatest risk of developing hepatic complications and cardiovascular disease [54–56].

3.1. Liver biopsy

As it was mentioned earlier, the chosen method to evaluate the grade of histological lesion is still the liver biopsy. However, liver biopsy has well-known limitations and cannot be proposed for all patients, given the high prevalence of NAFLD worldwide. Liver biopsy is invasive and is not without complications. Besides, there are other drawbacks: (1) sampling error, since a typical liver biopsy samples only 1/50,000 of all liver tissues, and histological lesions of NASH are unevenly distributed throughout the liver parenchyma [57]; (2) inter- and intra-observer variability, as observed by Gawrieh et al. although there was a high agreement ratio in the assessment of steatosis grading and fibrosis staging between pathologists, the agreement was suboptimal for lobular inflammation and hepatocellular ballooning [58]; and (3) the existence of different criteria for the definition of NASH. The Non-alcoholic Steatohepatitis Clinical Research Network (NASH-CRN) proposed the system termed the NAS scoring system in order to classify NAFLD according to severities of fatty change, inflammation and hepatocellular ballooning [3]. NAS is markedly reproducible and is useful for assessing therapeutic effects in Clinical trials, but it is incapable of diagnosing NASH in patients with burned-out NASH, in whom fatty changes and inflammatory cell infiltration resolving in fibrosis have progressed [59].

Given these limitations, non-invasive methods have been developed for the diagnosis of NASH and fibrosis as a first option to examine NAFLD patients and to help determine which require a liver biopsy. The ideal test should be economical, reproducible and capable of diagnosing the whole spectrum of lesions, including within NAFLD, and even reflecting the

changes produced on initiating specific treatment. Nowadays, we do not have a test available that has these characteristics, so these non-invasive methods are based on diverse complementary approaches: clinical factors, genetics, serological markers, image tests and transient elastography [60, 61].

3.2. Risk factors associated with non-alcoholic steatohepatitis and progressive disease

The best predictor of the evolution of NAFLD is the presence of necroinflammation and fibrosis in liver biopsy; however, there are more and more studies reporting no insignificant rates of progression of simple steatosis [5, 6, 62]. A first study that analyzed patients with NAFLD and paired biopsies demonstrated that even patients with simple steatosis can progress to NASH and advanced fibrosis, especially in the presence of metabolic risk factors [6]. Therefore, there is a series of non-modifiable and modifiable factors in patients associated with a greater risk of development of NASH and more progressive disease.

Various transversal studies have demonstrated that the disease is more severe in older patients, although this phenomenon could be due to the sum of pathogenic factors and a greater duration of the liver disease itself and the associated diseases [8, 63, 64]. In fact, the longitudinal studies have not managed to demonstrate that age is a factor that aggravates the disease per se [65]. The association between sex and fibrosis progression is controversial; two transversal studies show that men and post-menopause women have a greater risk of fibrosis in comparison with pre-menopause women; moreover, precocious menopause is associated with a greater risk of fibrosis [66–69]. Other non-modifiable factors are genetic; dozens of genes with multiple polymorphisms associated with NAFLD have been discovered thanks to genome-wide association studies (*GWAS*), but the number of strongly validated genes in large independent cohorts is limited to two, *patatin-like phospholipase domain containing 3* (PNPLA3) and *transmembrane 6 superfamily member 2* (TM6SF2) [70]. The presence of the single nucleotide polymorphisms (SNPs) rs738409 and rs58542926 of the genes PNPLA3 and TM6SF2, respectively, has been associated with a greater risk of NAFLD, as well as a more severe disease [71–76]. Recently, an SNP of IL28b (also implicated in the response to interferon in chronic hepatitis C patients (VHC)) has been associated with an increment in fibrosis in NAFLD patients [77]. Moreover, in a control-case study carried out by our working group, we have observed that the presence of the variants rs1421085 and rs1558902 of the fat mass and obesity-associated (FTO) gene confer a high risk of liver inflammation particularly in patients of normal weight with NAFLD (unpublished).

On the other hand, NAFLD tends to be more severe in patients with various factors of the metabolic syndrome, particularly DM2 and obesity. In fact, the reduction in weight and good glycemic control are associated with an improvement in inflammation and liver fibrosis [11, 78, 79]. However, it is known that NASH can also be present in slim subjects although it is unknown whether the natural history of the disease in these slim subjects is similar to that present in obese subjects. As for arterial hypertension, it is arguable whether its treatment improves the histology of NASH [5, 80]. Another factor of the metabolic syndrome, frequent in NAFLD patients, is dyslipidemia, fundamentally in the form of hypertriglyceridemia and atherogenic dyslipidemia [64, 81]; but moreover, a recent study has related the very low-density lipoprotein (VLDL) profile with the NAFLD severity,

observing that a decrease in small VLDL particle concentration is associated with more advanced fibrosis [82]. Vitamin D deficiency is also frequent among NAFLD patients, and its levels have been correlated negatively with the severity of steatosis, inflammation and fibrosis [83, 84].

Another possible factor associated with NAFLD progression is the alcohol consumption, a controversial aspect as despite there being a limit above which the consumption of alcohol would define alcoholic steatohepatitis (≥ 60 g/d in women and ≥80 g/d in men), it is not clear that we are confronting a pathology different to NASH given that the pathogeny of these entities presents a great similarity. Moreover, the quantification of alcohol consumption is quite subjective, imprecise, habitually underestimated and not contrasted with objective determinations through biomarkers. At present, there is no agreement on the impact of light-moderate consumption of alcohol on NAFLD given that the literature available about this topic shows contradictory results relate to NAFLD progression [85, 86]. Nevertheless, it seems that all the relevant studies are in favor of a possible benefit from the moderate alcohol consumption, defined as the consumption of up to one drink a day for women and two drinks a day for men [87]. While the consumption of large doses of alcohol leads to the development of insulin resistance and to the infiltration of macrophages into the adipose tissue [88], moderate consumption has been associated with an improvement in the sensitivity to insulin and high concentrations of adiponectin [89–91]. Various studies suggest a significant association between the moderate consumption of alcohol and the less histological severity of NAFLD [92, 93]. As for the development of HCC, only one prospective study exists that evaluates the consumption of alcohol with the risk of HCC in NAFLD, finding a greater risk of this tumor with moderate use of alcohol; however, this study is carried out in patients with cirrhosis due to established NASH, without evaluating the impact of alcohol on patients with a less severe disease [94].

3.3. Non-invasive diagnosis of non-alcoholic steatohepatitis

There is still no available image test in clinical practice capable of differentiating NAFL from NASH, so various biomarkers have been evaluated to predict the existence of NASH, which are related to pathogenesis pathways of the disease (apoptosis/cellular death, inflammation and oxidative stress).

The most studied serum biomarker associated with the presence of NASH is cytokeratin 18 fragments (CK18-F), a product of the degradation resulting from the apoptosis of hepatocytes mediated by caspase 3 [95], which is measured using enzyme-linked immunosorbent assay (ELISA). Various studies have demonstrated a significant increase in CK18-F in NASH patients in comparison with NAFL patients, and a positive correlation with fibrosis and the histological components of NASH [96, 97]. However, the sensitivity and specificity of this test are quite low, around 60% [98]. Oxidized low-density lipoprotein (LDL), thiobarbituric acid reactive substances (TBARS) and malonaldehyde have been used as markers of oxidative stress, but the results are contradictory [99, 100]. Among the markers of inflammation studied include leptin, protein C reactive, interleukin 6, hyaluronic acid, adiponectin and tumor necrosis factor α (TNFα). All of them have been evaluated in short series or pilot studies in heterogeneous groups of patients with contradictory results [101].

With the aim of improving the diagnostic value of the biomarkers, predictive models have been developed that combine some of these serum biomarkers with analytical parameters and clinical variables, but they have not been adequately validated, so up to now, they are not recommendable in clinical practice [102–107] (**Table 2**).

3.3.1. Emerging fields

Emerging fields in the search for non-invasive biomarkers of NAFLD are proteomics, metabolomics and epigenetics.

Proteomics provides essential information about the biologically active entity named protein. Thanks to proteomic analysis, key changes in serum protein expression levels have been demonstrated between control subjects and patients with different stages of fatty liver [108].

In the last years, studies about the use of metabolomic to discover biomarkers of progression of NAFLD have received great interest, and not only in this liver disease [109–111]. In fact, a Spanish group has developed the so-called OWL Liver Test that consists in the determination

Model	Variables	Sensitivity (%)	Specificity (%)
HAIR score	Hypertension, ALT, insulin resistance	80	89
NASHTest	Age, gender, weight, height, cholesterol, triglycerides, α2-macroglobulin, apolipoprotein A1, haptoglobin, GGT, ALT, AST, bilirubin	88	50
NASH score	PNPLA3 genotype, insulin, AST	75	74
Nice model	Ck18, ALT, metabolic syndrome	84	86
NAFLD diagnostic panel	Diabetes, gender, BMI, triglycerides, M30, M65-M30	91	47
OxNASH	Age, BMI, AST, 13-Hydroxyoctadecadienoic acid, linoleic acid	81	–

Table 2. Predictive models for non-alcoholic steatohepatitis.

of more than 500 serum metabolites through liquid chromatography coupled with mass spectrometry (LC–MS) in NAFLD patients obtaining a metabolomic profile that enables the differentiation between NAFL and NASH with good specificity and sensitivity [112]. Moreover, the same group thanks to the study of metabolomic profiles at the serum level observed two different subtypes of NAFLD according to the involvement of the methionine metabolism, subtype M and subtype no M, distinguishing those patients that could benefit from therapy with SAMe (S-adenosyl methionine) [113].

Recently, studies in rodents suggest that epigenetic events, inheritable events not caused by changes in DNA sequence, may influence susceptibility to NASH. The three most commonly described epigenetic mechanisms are DNA (CpG) methylation, post-translational histone modifications and microRNAs (miRNAs). Several miRNAs have been identified in serum/plasma of NAFLD patients that show diagnostic potential for distinguishing NAFL from NASH and advanced fibrosis [114].

4. Diagnosis of hepatic fibrosis

The stage of fibrosis ranges from absent (F0) to cirrhosis (F4), with stages F2–F4 considered to be clinically significant and stages F3–F4 considered to be advanced fibrosis. Apart from liver biopsy, there are two broad categories of non-invasive markers used to determine the stage of liver fibrosis: serum and radiological markers. This stratification based on markers of fibrosis is more tractable than those used for NASH and so it is currently used to identify patients who are at risk of disease progression.

4.1. Serum biomarkers

There are two large groups of predictive models of advanced fibrosis: 'simple bedside models', which use a combination of routine blood tests and clinical variables, and 'complex models', which use serum markers of fibrosis (measures of extracellular matrix deposition and turnover).

Although several of these predictive models of advanced fibrosis have been evaluated (**Table 3**) [61, 66, 115], two of the tests have been more widely studied and have easily available parameters, *Fibrosis-4 index* (FIB-4) and *NAFLD Fibrosis Score* (NFS). FIB-4 is based on age, levels of AST, ALT and platelet count. Values of this index below −1.30 enable the exclusion of the presence of advanced fibrosis with a sensitivity of 74% and a specificity of 71%, while values above 2.67 indicate advanced fibrosis with a sensitivity and specificity of 33 and 98%, respectively [115]. NFS is another formula developed and validated for the detection of advanced fibrosis that includes age, BMI, presence of diabetes or hyperglycemia, platelet count, albumin and AST/ALT ratio (http://nafldscore.com/). In a meta-analysis of 13 studies with more than 3000 patients, a value of NFS < −1.455 had a sensitivity of 90% and a specificity of 60% to exclude advanced fibrosis, while a value of >0.676 identified the presence of it with a sensitivity of 67% and a specificity of 97% [116].

Model	Variables	Cut-offs	Sensitivity (%)	Specificity (%)
FIB-4	Age, AST and ALT	1.30 (low cut-off)	74	71
	levels, platelets	2.67 (high cut-off)	33	98
NAFLD	Age, hyperglycemia,	−1.455 (low cut-off)	90	60
fibrosis score	BMV, AST/ALT ratio,	0.675 (high cut-off)	67	97
	albumin, platelet			
AST to	AST, platelet	1	27	89
platelet ratio				
index (APRI)				
AST/ALT ratio	AST, ALT	0.8 (low cut-off)	90	60
		1 (high cut-off)	67	97
BAAT score	BMI, age, ALT, serum	2	71	80
	triglycerides			
BARD score	BMI, AST/ALT ratio,	2	89	44
	diabetes			
Enhanced	Age, hyaluronic acid,	8.5 (low cut-off)	80	90
liver fibrosis	TIMP-1, PIIINP	11.3 (high cut-off)		
(ELF) test				
FibroTest	α2-macroglobulin,	0.3 (low cut-off)	77	77
	haptoglobin, GGT,	0.7 (high cut-off)	15	90
	bilirubin,			
	apolipoprotein			
Hepascore	Age, gender, bilirubin,	0.44	75	84
	hyaluronic acid, α2-			
	macroglobulin			

AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; TIMP-1, tissue inhibitor of metalloproteinase *1*; PIIINP, procollagen III amino-terminal peptide; GGT, gamma glutamyl transferase.

Table 3. Predictive models for significant and advanced fibrosis in NAFLD patients.

The principal drawback of all these biomarkers is that none of them is specific of the liver and their results can be influenced by co-morbidities of patients, so a critical interpretation of the result is necessary.

4.2. Imaging methods to measure fibrosis

With respect to image techniques, transient elastography (FibroScan®) is the most widely used technique in the diagnosis of liver fibrosis, not only in NAFLD but also in different chronic

liver diseases [117]. TE measures the propagation velocity of low-frequency waves (50 Hz) through the hepatic parenchyma using ultrasounds and is expressed in kilo Pascal (kPa); the higher the propagation velocity, the greater the stiffness of the tissue. The advantages provided by this technique are its speed, the immediacy of the results and the ease of handling. However, proper results require careful interpretation of data, based on at least 10 successful measurements, a success rate above 60% and an interquartile range (IQR) of <30% of the median value. A limitation of TE in NAFLD is the high rate of technical failure due to the attenuation of the elastic wave by interposition of adipose tissue secondary to the central obesity, very frequent in these patients. Although an XL probe has been developed, which enables greater penetration of the wave, this difficulty is often insurmountable [118, 119]. Moreover, this technique has been initially validated in patients with chronic infection by VHC [120], while the studies focusing on evaluating its use in NAFLD are smaller and have often used different cut-offs [42, 118, 121–129] (**Table 4**). According to the results of several studies, the cut-offs with M probe accepted for NAFLD patients are 7.0 kPa for significant fibrosis (≥F2), 8.7 kPa for advanced fibrosis (≥F3) and 10.3 kPa for cirrhosis (F4) [124, 126, 128]. When using the XL probe, these cut-offs differ as the measure of liver stiffness with this probe is less than that with the M probe in the same patient; in this case, 6.2, 7.2 and 7.9 kPa are the cut-offs for significant fibrosis, advanced fibrosis and cirrhosis, respectively [119, 130, 131].

Another liver elasticity-based imaging technique is ARFI (*acoustic radiation force impulse imaging*). Although for the time being there are few studies that have evaluated its utility in NAFLD patients, its great advantage is that it can be easily connected to traditional ultrasound scan enabling the positioning of the zone of interest under visual control [132, 133]. Another method suitable for studying the elastic properties of the hepatic parenchyma is magnetic resonance elastography (MRE). MRE can be more reliable than TE to diagnose advanced fibrosis; moreover, it has the advantage of being able to evaluate the whole hepatic parenchyma even in obese patients, but the technique is expensive and not widely available [134, 135]. Magnetic resonance imaging is more widely available and is the basis of new software called DEMILI (*Detection of Metabolic-Induced Liver Injury*), which through computerized optical analysis of its images determines a series of optical biomarkers enabling the detection of the presence of NASH (NASHMRI) and predicting significant fibrosis (FibroMRI) in NAFLD patients. For the detection of NASH, a cut-off has been established with NASHMRI of >0.5, presenting a sensitivity and specificity of 87 and 60%, respectively. In the case of FibroMRI, the cut-off is also >0.5 for the prediction of significant fibrosis with a sensitivity of 77% and a specificity of 80% [136]. Given that this technique enables the analysis of the total volume of the liver, as well as its use in the diagnosis of NASH and significant fibrosis, it enables the potential effects of a therapy to be monitored.

A recently developed technique is multiparametric magnetic resonance (MR) that includes T_1 mapping for fibrosis/inflammation imaging, T_2* mapping for liver iron quantification and proton magnetic resonance spectroscopy (^{1}H-MRS) for liver fat quantification. In a recent study, it has demonstrated good correlation with disease severity in NAFLD patients, showing excellent accuracy in quantifying both the inflammatory and fibrotic components of NAFLD [137].

A summary of the approach to the management and characterization of NAFLD patients is shown in **Figure 1**.

Study	Patients, *n*	Probe	Fibrosis Stage	Cut-off (kPa)	Sensitivity (%)	Specificity (%)
Yoneda et al. [122]	67	M	$F \geq 2$	6.65	82	91
			$F \geq 3$	8.0	87	84
			$F = 4$	17.0	100	98
Yoneda et al. [125]	97	M	$F \geq 2$	6.65	74	97
			$F \geq 3$	9.8	85	81
			$F = 4$	17.5	100	97
Nobili et al. [129]	52	M	$F \geq 2$	7.4	100	92
			$F \geq 3$	10.2	100	100
			$F = 4$	–		
Wong et al. [128]	246	M	$F \geq 2$	7.0	79	76
			$F \geq 3$	8.7	84	83
			$F = 4$	10.3	92	88
Lupsor et al. [124]	72	M	$F \geq 2$	6.8	67	84
			$F \geq 3$	10.4	100	97
			$F = 4$	–		
Petta et al. [118]	169	M	$F \geq 2$	7.25	69	70
			$F \geq 3$	8.75	76	78
			$F = 4$	–		
Kumar et al. [126]	205	M	$F \geq 2$	7.0	78	79
			$F \geq 3$	9.0	85	88
			$F = 4$	11.8	90	88
Pathik et al. [121]	110	M	$F \geq 2$	9.1	–	–
			$F \geq 3$	12.0	90	80
			$F = 4$	20.0	90	80
Cassinotto et al. [123]	291	M	$F \geq 2$	6.2	90	–
			$F \geq 3$	8.2	90	–
			$F = 4$	9.5	90	–
Imajo et al. [42]	142	M	$F \geq 2$	11.0	62	100
			$F \geq 3$	11.4	86	84
			$F = 4$	14.0	100	76

Table 4. Comparative studies of FibroScan with liver biopsy in the detection of fibrosis in NAFLD.

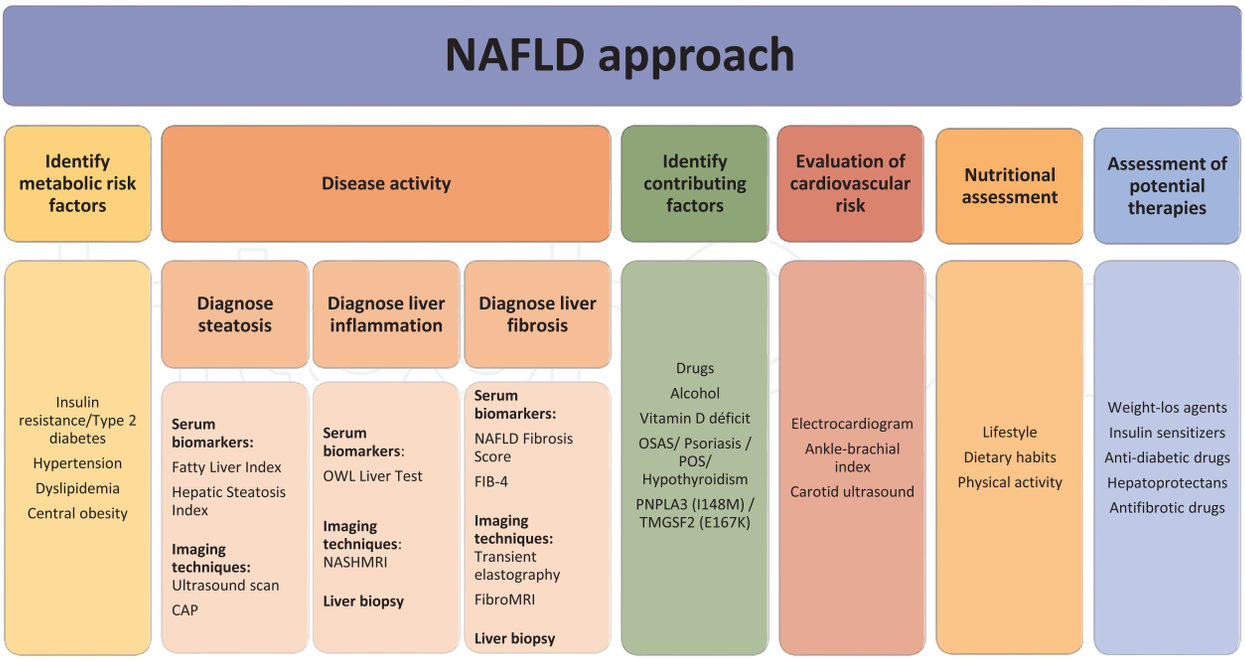

Figure 1. Practical approach to the management of patient with NAFLD.

5. Monitoring disease progression

A recent meta-analysis of 11 studies that evaluated the progression of NAFLD through the use of paired liver biopsies revealed that patients with NAFL and NASH presented a progression of fibrosis of 33.6% and an improvement of it of 22.3%, the rate of fibrosis progression being greater in patients with NASH than with NAFL (progression in a stage of 7.1 years compared to 14.3 years, respectively) [5]. However, there is a lack of homogenization in the speed of fibrosis progression in all these studies with paired biopsy, which is mostly due to the presence of characteristics of the metabolic syndrome in the patients [5–7, 62, 65, 138–144]. For this reason, due to the lack of studies that provide complete data about the differential progression of the disease in patients with different stages of NAFLD, there is no guide on the frequency of follow-up of these patients or on the means available to monitor the progression. Nevertheless, once the diagnosis of NAFLD is made, the follow-up will depend on the presence of metabolic risk factors and the severity of the hepatic disease, which will be determined by the presence of NASH and, especially, the stage of fibrosis.

The principal metabolic factor of the risk of NAFLD progression is DM2 [1, 19, 145]. Patients with DM2 have a more severe grade of NAFLD than the patients without DM2, with rates of NASH up to 80% and of advanced fibrosis of 30–40% [146, 147]. These data confirm the need for closer monitoring in these patients. Bazick et al. [147] developed a clinical model to detect NASH and advanced fibrosis in patients with NAFLD and DM2 with a sensitivity and specificity of 57 and 90%, respectively. This model includes easily accessible parameters such as BMI, circumference at the waist, HbA1c, insulin resistance, ALT, AST, albumin and ferritin, for NASH; and age, BMI, waist/hip ratio, arterial hypertension, ALT/AST ratio, alkaline

phosphatase, bilirubin, globulin, albumin, serum insulin, hematocrit, INR and platelets, to predict advanced fibrosis. However, further studies are still necessary to externally validate this model. Other metabolic factors described with more evidence for the disease progression are central obesity, arterial hypertension and high levels of LDL cholesterol [7, 148–150]. No study shows cost-efficacy in the monitoring of the progression in these at-risk patients, but we recommend carrying out NFS and/or FIB-4 every 2 or 3 years in these patients with non-significant fibrosis, and if NASH and/or significant fibrosis is presented in the initial diagnosis, the follow-up will not differ from the rest of the patients.

The other factor having the greatest effect on the disease progression is liver fibrosis [151]. In general, in a period of 15 years, 13% of the patients with stage F2 and 25% of those presenting with F3 will develop cirrhosis [6, 7, 62]. These patients with significant fibrosis should be considered for pharmacological treatment, besides lifestyle modifications (diet and exercise). Moreover, NAFLD patients may develop HCC even in the absence of cirrhosis [152], given that it is the continuous hepatocyte injury that leads to a compensatory proliferation, key driver of the development of HCC [153]. Therefore, patients with NASH and significant fibrosis, which is indicative of important cellular damage, are also at risk of developing this liver tumor.

With all this information, we recommend recalculating NFS and/or FIB-4 every 4–5 years for patients with NAFL without risk factors or if the patient develops DM2; in patients with NASH without significant fibrosis, we recommend an annual follow-up with a calculation of NFS and/or FIB-4 and carrying out TE and ultrasound, and in patients with significant fibrosis, a 6-monthly follow-up is recommended with special interest in screening for HCC. The management and follow-up of the patients with advanced fibrosis/cirrhosis due to NASH does not differ from the rest of etiologies [25].

Another important question is the evaluation of the response to the therapy provided. The non-invasive methods available currently have not been reliable or have not been validated to document efficacy of the treatments, so liver biopsy is still necessary to determine this efficacy, especially in a clinical trial setting.

6. Screening of associated diseases

In recent years, several studies have confirmed that the morbimortality associated with NAFLD is not limited only to hepatic injury, yet it is a disease with multisystemic behavior with affectation of different organs.

6.1. Insulin resistance and metabolic syndrome

As was previously mentioned, the concurrent characteristics of metabolic syndrome increase the risk of developing NAFLD, and a recent study of the HepaMet group relates the severity of NAFLD with the number of factors of the metabolic syndrome present (publication pending). However, the presence of NAFLD in itself also increases the risk of developing complications such as dyslipidemia and insulin resistance [154–156]. In this sense, the diagnosis and

quantification of hepatic fat can be useful in the prediction of future development of diabetes and other cardiovascular risk factors [56].

Insulin resistance is a key in the physiopathology of NAFLD, associated with the increase in the deposit of fat and fibrosis, and it substantially increases the risk of developing DM2, which indicates that NAFLD can precede the development of diabetes. Moreover, and as it was mentioned earlier, several studies have demonstrated that, especially in patients with insulin resistance and/or diabetes, liver fibrosis can progress even when a baseline hepatic histology described only simple steatosis without hepatocellular damage [62, 141]. All in all, in daily clinical practice the use of screening tools is necessary to detect the presence of diabetes (fasting blood glucose levels, HbA1c or, if available, the oral glucose tolerance test) or insulin resistance. The reference technique for the diagnosis of IR in non-diabetic patients is the hyperinsulinemic-euglycemic clamp test, although this procedure is expensive and complicated, so it is not routinely used in daily clinical practice [157]. In these cases, the calculation of HOMA-IR (*homeostatic model assessment*) is an acceptable alternative to evaluate the IR, although there is no agreement on the threshold that defines insulin resistance using this formula [158]. Nevertheless, HOMA-IR can help us during the follow-up to identify patients at risk of fibrosis progression [6, 62]. The next question once the patients with IR are identified is whether it is necessary to treat them pharmacologically or not; and, whether in diabetic patients is necessary to intensify the anti-diabetic treatment to avoid liver disease progression or not. As expected, several insulin-sensitizing agents have demonstrated an improvement in the hepatic histology [159–161], even in patients without DM2 [162, 163], given that both entities share multiple physiopathological mechanisms, so this treatment can be considered in patients with NASH and/or multiple factors of progression in which a decrease of IR cannot be achieved with diet and exercise, although the EASL and AASLD guidelines do not contemplate it. Given that IR plays an essential role in NAFLD progression but not the only one, we do not believe that it is necessary to treat DM2 differently/intensely in patients with NAFLD, independently of the grade, provided that the IR is controlled.

6.2. Cardiovascular disease

Cardiovascular disease is quantitatively the main cause of death in NAFLD patients. Besides the risk itself of the characteristics of the metabolic syndrome, multiple pathogenic conditions of NAFLD contribute to the development of cardiovascular disease. In fact, patients with NAFLD often present elevation in the markers implicated in the development of atherosclerosis, such as CD36 in its soluble form (sCD36), a membrane receptor responsible for, among other things, the transport of fatty acids [164]. The spectrum of CVD in NAFLD includes atherosclerotic coronary heart disease, heart failure and cardiac arrhythmias. This necessitates the study of probable CVD, especially subclinical atherosclerosis, in all these patients [10]. There are little data to define the optimal means of screening NAFLD patients with CVD, but it is important to be aware that there are different techniques for the detection of subclinical atherosclerosis that are bloodless and some of which are very easily performed. Among these, the measurement of ankle-brachial index and carotid ultrasound are assessments especially useful for patients with intermediate cardiovascular risk, situation affecting a very important part of the population with NAFLD [165].

6.3. Extrahepatic cancer

The second most prevalent cause of death among patients with NAFLD is cancer, both gastrointestinal (colon, esophagus, stomach and pancreas) and extraintestinal (kidney and breast), which leads to the suspicion that this liver disease might promote the development of neoplasms.

The association of insulin resistance/diabetes, obesity and metabolic syndrome with an increase in the risk of a large number of cancers is well established [166–171]. These three characteristics are closely related to NAFLD and contribute significantly to the risk of developing HCC; nevertheless, various recent studies indicate that NAFLD can be an additional and independent risk factor for extrahepatic cancers [172, 173], especially colorectal cancer (CRC) [127, 174]. In several studies, colorectal lesions, particularly tubular adenomas and carcinomas, were significantly more prevalent in NAFLD patients, regardless of age, sex and manifestations of metabolic syndrome; even the presence of NASH has been related to a greater risk in comparison with those with NAFL [174, 175]. This rise in the risk of CRC in NAFLD can be explained by the increase in insulin and pro-inflammatory cytokines and the alteration of the adipokines metabolism predominantly leptin versus adiponectin that exists in these patients and which promotes cellular proliferation, inhibition of apoptosis and angiogenesis [176, 177].

Although these data clearly suggest more rigorous screening programmes for CRC in NAFLD patients, there are no well-designed prospective studies enabling the verification of a causal relation between NAFLD and CRC or studies that evaluate the usefulness of earlier screening in this liver disease, so no guidelines make a distinction with respect to CRC screening in these patients.

6.4. Other associated diseases

There is increasing interest in the possible contribution of NAFLD to the development and progression of chronic kidney disease (CKD) [178–181]. A recent meta-analysis has revealed that the presence and severity of NAFLD are associated with an increase in the risk and severity of CKD [181]. However, it is difficult to establish NAFLD as an independent risk factor of CKD given the close relation between NAFLD and other known risk factors of CKD such as obesity and IR. Obstructive sleep apnea syndrome (OSAS) is strongly associated with NAFLD independently of other traditional factors; it is a consequence of the decrease in the lipid metabolism provoked by intermittent hypoxia [182–185]. Other described diseases associated with NAFLD include osteoporosis [186], psoriasis [187], polycystic syndrome [188] and other endocrinopathies such as hypothyroidism [189], hypopituitarism [190] and hypogonadism [191]. Until now, there is no evidence for screening of all these pathologies for the mere fact that the subject presents NAFLD, so all that needs to be studied is the presence of them if the patient has clinical manifestations related to them. Moreover, a recent study by our group has demonstrated that psychotic patients with specific pharmacological treatment have a high risk of developing NAFLD in the first years, so its early detection will enable better prevention of cardiovascular events, which are so increased in this population [192].

7. Diagnostic algorithm and follow-up

While working on the different sections of this chapter, we have detailed the fundamental elements for the development of a diagnostic algorithm and a follow-up procedure for NAFLD (**Figure 2**). This algorithm is based on clinical evidence available in the current literature with respect to the topic and on different guidelines issued by the principal international associations for the study of the liver (EASL and AASLD). In the case of monitoring and follow-up of these patients where the existing evidence is not relevant in certain aspects, our recommendations are based on the experience of our clinical group in different high-quality studies in this field.

Once the initial diagnosis of NAFLD is made, our posterior attitude will depend on the result of the non-invasive liver fibrosis methods. In general, current image techniques are quite reliable to distinguish between advanced fibrosis (≥*F*3) and mild fibrosis or null (*F*0–*F*1), but they are insufficient to identify those patients with significant fibrosis (≥*F*2). Therefore, in clinical practice, we recommend the combination of elastographic techniques with serum markers, more specifically TE and NFS due to their wide accessibility and ease of application. When these two parameters generate doubt about the grade of fibrosis or indicate possible significant fibrosis, liver biopsy is necessary. Depending on the result, we determine the posterior follow-up as can be seen in the algorithm (**Figure 2**). The presence of metabolic risk factors influences not only the therapeutic management but also the follow-up. If liver biopsy is

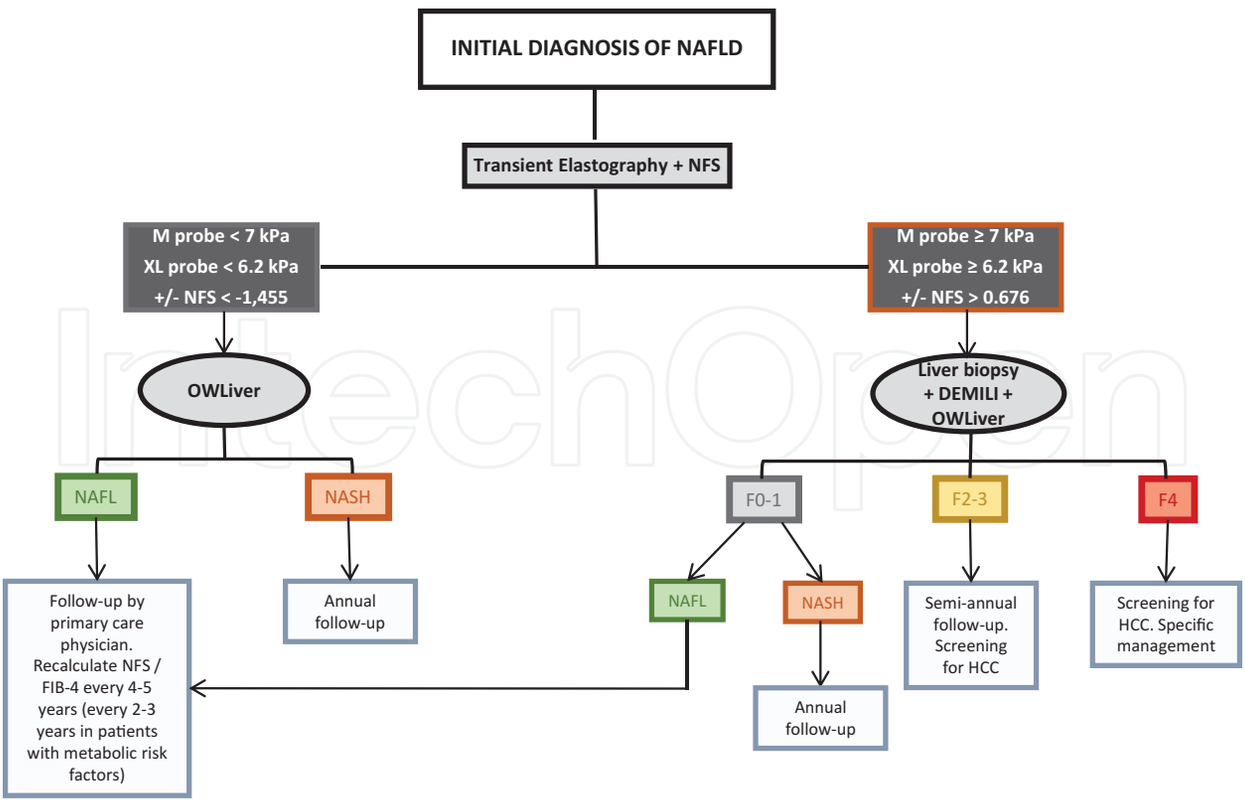

Figure 2. Clinical algorithm for the diagnosis of NAFLD and monitoring disease progression.

not to be performed on the patient with NAFLD, due to advanced age, to the absence of significant fibrosis in the non-invasive methods or to contraindication, we could evaluate the performance of the OWL Liver Test to help identify those patients with NASH who require a closer follow-up. If the patient does not present improvement in laboratory parameters even in imaging tests, we should evaluate to repeat liver biopsy 5 years after the last one, or even before if progression of the disease is suspected.

8. Conclusions

NAFLD is currently the primary cause of chronic liver disease in the western world and its growth is a consequence of its close relation to obesity and metabolic syndrome. One of the great challenges in this disease is to diagnose and classify it correctly, given that the characteristics defining NAFLD are the common denominator of many liver diseases. Its correct characterization is important as in spite of presenting a generally benign and slowly developing evolution from the hepatic viewpoint; the fatty liver can progress towards more severe forms with the development of inflammation, fibrosis, cirrhosis and HCC, thus conferring morbimortality. However, its potential morbimortality is not limited to this organ, but goes beyond; NAFLD is being considered a mediator of systemic diseases. Therefore, the early identification of these patients would help to improve its prognosis through an individualized intervention depending on the stage of liver disease, on the metabolic risk factors present and on the cardiovascular risk, which translates into the need for a systemic approach to the disease with multidisciplinary management including primary care physician, endocrinologists, nutritionists, psychologists and hepatologists.

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this chapter.

Author details

Paula Iruzubieta, Marta González, Joaquín Cabezas, María Teresa Arias-Loste and Javier Crespo*

*Address all correspondence to: javiercrespo1991@gmail.com

Department of Gastroenterology and Hepatology, Marqués de Valdecilla University Hospital, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Infection, Immunity and Digestive Pathology Group, Research Institute Marqués de Valdecilla (IDIVAL), Santander, Spain

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