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TOPIC HIGHLIGHT

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Noninvasive investigations for non alcoholic fatty liver disease and liver fibrosis

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Abstract

Non-alcoholic fatty liver disease (NAFLD) includes a spectrum of diseases that have insulin resistance in common and are associated with metabolic conditions such as obesity, type 2 diabetes mellitus, and dyslipidemia. NAFLD ranges from simple liver steatosis, which follows a benign course, to nonalcoholic steatohepatitis (NASH), a more severe entity, with necroinflammation and fibrosis, which can progress to cryptogenic cirrhosis and end-stage liver disease. Liver biopsy remains the gold standard for evaluating the degree of hepatic necroinflammation and fibrosis; however, several noninvasive investigations, such as serum biomarkers, have been developed to establish the diagnosis and also to evaluate treatment response. These markers are currently neither available in all centers nor validated in extensive studies. Examples include high-sensitivity C reactive protein and plasma pentraxin 3, which are associated with extensive liver fibrosis in NASH. Interleukin-6 correlates with inflammation, and cytokeratin-18 represents a marker of hepatocyte apoptosis (prominent in NASH and absent in simple steatosis). Tissue polypeptide specific antigen seems to have a clinical utility in the follow-up of obese patients with NASH.

Key words: Non-alcoholic fatty liver disease; Biomarkers; Necroinflammation; Liver fibrosis

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) represents a group of conditions ranging from simple liver steatosis, usually asymptomatic, to nonalcoholic steatohepatitis (NASH), which is characterized by the presence of apoptosis/inflammation and fibrosis, and also by a progressive course, evolving to cryptogenic cirrhosis.

Non-alcoholic steatohepatitis (NASH) was first described in 1980 by Ludwig *et al*[1] in patients with abnormal liver tests and fatty infiltration accompanied by inflamma-

tory changes at histological examination. Prior to 1980, hepatic steatosis was documented in patients with associated metabolic conditions, especially in obese patients who underwent liver biopsy before and after bariatric surgery[2].

The prevalence of NAFLD has increased over the last two decades and it affects approximately 30% of adults in the United States[3] and almost a third of the general population[4]. The most common form of NAFLD encountered in clinical practice is liver steatosis, also known as non-alcoholic fatty liver (NAFL), if it occurs in the absence of significant alcohol consumption (more than 10 g/d in females and 20 g/d in men). Data analysis from two recent prospective cohort studies concluded that raised body mass index (BMI) and alcohol consumption are both related to liver disease, with evidence of a supra-additive interaction between the two[5]. The study by Liu *et al*[6] confirmed that excess body weight increases the incidence of liver cirrhosis. In middle aged women in the UK, an estimated 17% of incident or fatal liver cirrhosis is attributable to excess body weight, as compared with an estimated 42% attributable to alcohol[6].

Fatty liver disease has a benign clinical course, as long as inflammatory injury of the liver does not develop. It is essential to differentiate between this form of simple steatosis, which is associated with a favorable long-term prognosis, and NASH, with a different natural history; approximately 20% of patients with NASH will develop cryptogenic cirrhosis and even end-stage liver disease[7]. Cirrhosis associated with NASH might even progress to hepatocellular carcinoma[8] and death related to NASH was reported to be approximately 12%-25% over a 7-10 years period[9]. NAFLD is an important cause of cryptogenic cirrhosis, as Powell *et al*[7] suggested, although other disorders could progress to this type of cirrhosis[10]. Another important issue concerning NAFLD is related to the inner mechanisms of the disease. Even though steatosis is a benign condition and NASH a progressive one, the basic mechanisms of both entities seem to be the same. This is supported by the study of Tarantino *et al*[11] who reported similar levels of transforming growth factor-β1 in serum of patients with simple steatosis and those with NASH.

PATHOGENESIS OF NAFLD

The pathogenesis of NAFLD, and especially of NASH, is not completely understood; however, a few mechanisms were proposed to explain the liver injury associated with metabolic syndrome[12]. Identification of these mechanisms has therapeutic importance, because targeted therapies might prevent the progression of NAFLD to fibrosis and cirrhosis[13].

Insulin resistance plays a central role in NASH pathogenesis. Insulin resistance is the main feature of metabolic syndrome, which embodies obesity, hypertension, diabetes, and dyslipidemia[14]. NAFLD is considered to be the hepatic component of metabolic syndrome[15-17]. Although overweight and obesity are present in the majority of patients with NASH, steatohepatitis can also occur in subjects with normal body weight[18]. A direct correlation between the degree of obesity and NASH development has been observed[19]; however, not all obese patients will have NAFLD. Hepatic steatosis evidenced by ultrasound is clearly more pronounced in cases of insulin resistance compared with healthy subjects[20]. Insulin resistance promotes disturbances in lipid metabolism, with increased delivery of free fatty acids to the liver, impaired mitochondrial β oxidation, "*de novo*" lipogenesis, and decreased β export from the liver[19], all of which result in fatty liver development. Some authors suggest that hyperinsulinemia of NAFLD is the result of the decreased insulin extraction by the liver[21,22]. NASH is also associated with mitochondrial abnormalities, such as swollen or elongated mitochondria with crystalline inclusions[18,23]. Liver overloading with lipids initiates multiple pathways including lipid peroxidation, generation of reactive oxygen species, oxidative stress, and production of inflammatory cytokines. In fact, oxidative stress is a trigger for lipid peroxidation in hepatocytes, with subsequently secretion of proinflammatory cytokines and activation of fibrosis-developing stellate cells, which are the main mediators of liver fibrosis. tumor necrosis factor (TNF)-α is increased in NAFLD patients and it has a central role in liver injury and disease progression from fatty liver to steatohepatitis and hepatic fibrosis, by activating both Kupffer and stellate hepatic cells[12,24]. Taking into account this hypothesis, targeted therapies against TNF-α might be beneficial in NASH treatment[25,26].

Another concept involves adipocytokines, which are secreted by the adipose tissue (WAT) known as white adipose tissue and are related to visceral obesity. WAT is responsible for secretion of a several adipokines and cytokines, such as adiponectin, leptin, TNF-α, and interleukin (IL)-6, which are involved in hepatic inflammatory process[27,28].

A theory concerning iatrogenic NAFLD induced by several medications has emerged. Drugs like diltiazem, amiodarone, tamoxifen, steroids, and antiretrovirals are involved in fatty liver or insulin resistance development[18].

Besides the well-recognized risk factors for NAFLD, such as type 2 diabetes, insulin resistance, hyperlipemia, and obesity, other metabolic conditions have been associated with fatty liver disease, namely polycystic ovary syndrome, and lipodystrophy[29,30]. Other rare conditions associated with NAFLD are hypobetalipoproteinemia, Weber-Christian syndrome, total parenteral nutrition, toxic exposure at organic solvents, dimethylformamide, gastric by-pass, and jejunoileal bypass[18].

DIAGNOSTIC PROCEDURES

The goal of diagnostic procedures is to identify the patients with NASH before the onset of advanced fibrosis. Liver biopsy is now considered the "gold standard" for the assessment of liver fibrosis. It is important to take into account that a needle biopsy is merely a sample of the entire liver and that fibrosis presents a diffuse pattern in chronic liver disease. The liver biopsy removes only about 1/50 000th of the liver and carries substantial interpretation errors. Liver biopsy is an invasive procedure with certain unavoidable risks and complications[3].

Therefore, the development of noninvasive tests for assessing hepatic inflammation and fibrosis has become an active area of research.

NAFLD is first of all a diagnosis of exclusion, so other

specific causes of liver diseases should be ruled out: viral hepatitis, alcoholic liver disease, Wilson disease, hemochromatosis, and autoimmune hepatitis[15]. The most challenging of them is exclusion of alcoholic liver disease, because the histological picture of both conditions is similar. It is necessary to obtain an accurate history concerning the patient's daily alcohol intake; knowing that consumption of more than 10 g/d in females and 20 g/d in males are responsible for liver injury in the absence of other risk factors, such as obesity, diabetes, and viral hepatitis[30].

The clinical presentation of patients with either NAFLD or NASH is proteiform. The majority of subjects are asymptomatic, but some of them can present with fatigue or right upper quadrant discomfort. Hepatomegaly is discovered in 50% of patients at physical examination[31]. The presence of fatigue does not correlate with the severity of liver injury[31]. These patients share a common clinical feature, obesity, and potentially other features of metabolic syndrome: hyperglycemia, dyslipidemia, and hypertension. Liver dysfunction might be discovered incidentally during a routine check-up, or a work-up for other conditions (Table 1).

Table 1 Clinical features of nonalcoholic fatty liver disease

Symptoms	Signs
None	Hepatomegaly (50% of patients)
Fatigue	Obesity
Right upper quadrant discomfort	Hypertension

Laboratory tests

Approximately 80% of patients with NAFLD have liver function tests in normal ranges; only a small proportion exhibits mild elevation of aminotransferases[32]. The ratio between aspartate aminotransferase (AST) and alanine aminotransferase (ALT) is predictive for the severity of the liver disease, with an AST/ALT ratio > 1 suggesting cirrhosis or advanced fibrosis[33,34]. The degree of aminotransferases elevation is not higher than four times of the upper limit of normal and does not correlate with the severity of steatosis or fibrosis[18]. In the majority of cases, ALT/AST ratio is > 1.

Can serum aminotransferases levels distinguish between NASH and NAFLD? Higher AST and ALT levels, and AST/ALT ratio are all significantly associated with NASH. Serum AST presents a stronger association and has a higher likelihood of discriminating NASH from other forms of NAFLD. However, a multivariable model using both AST and ALT, showed that the discriminating score for distinguishing the patients with NASH from those without NASH was only 26%. This result indicates that additional noninvasive methods are needed for an accurate diagnosis. Fracanzani *et al*[35] studied 455 patients with NAFLD, divided in two groups according to their serum ALT levels. They compared clinical and histological features of patients with and without increased serum ALT. NASH was diagnosed in 62% and 74% of patients with normal or increased ALT levels, respectively. There were no significant differences in advanced fibrosis between the two groups, underlying the need for liver biopsy for diagnosis and staging of fibrosis in NASH[35].

Laboratory signs of advanced liver disease, such as hyperbilirubinemia, hypoalbuminemia, and abnormal prothrombin time are seen only in cases associated with cirrhosis. Other biological abnormalities, such as hyperglycemia and hypertriglyceridemia are related to the coexistent metabolic conditions.

Laboratory assessment of dyslipidemia and insulin resistance should also be performed. A simple laboratory test was designed to evaluate the insulin profile. It is known as homeostasis model assessment (HOMA), and is defined as the fasting insulin level (μU/mL) multiplied by the fasting glucose level (mmol/L) and divided by 22.5[20]. Although HOMA is not a perfect measure of insulin resistance, it is an easy way to estimate insulin resistance. A possible link between HOMA and hepatic steatosis has been demonstrated[20].

The major problem remains to determine the consequences of small amounts of alcohol intake in a patient with liver disease. Taking into account the difficulty of distinguishing between alcoholic and nonalcoholic liver disease based on patient's history, many attempts have been made to assess alcohol consumption using serum markers. Over time, several surrogate markers for alcoholism have been determined: high serum concentration of γ-glutamyltransferase, increased mean corpuscular volume, increased AST levels, AST/ALT ratio > 2, and a desialylated transferrin/total transferrin ratio > 1[18,36].

NAFLD is also accompanied by changes in serum iron markers, such levels of ferritin in 20%-50% of patients and increased transferrin saturation in 5%-10% of cases[33]. Hemochromatosis gene testing is recommended if the ferritin level is significantly elevated.

Overall, none of these tests have specificity for the diagnosis of NAFLD, pointing out only a liver dysfunction. The pattern of aminotransferase elevation does not provide an etiological clue for the hepatic disease, nor does it make a distinction between simple fatty liver and NASH[18].

In fact, the differentiation between steatosis and steatohepatitis can be made only by a histological approach[33]. Besides, the amount of lipid accumulated in the liver cannot be assessed using functional liver tests; however, the degree of liver infiltration with fat can be diagnosed using a variety of imaging methods.

Imaging studies

The most common and less invasive imaging technique used for NAFLD diagnosis is ultrasonography. Ultrasonography, the first-line imaging technique, assesses the presence of steatosis, showing a hyperechogenic liver parenchyma, known as "bright liver" and "blurring of the vascular margins". The increased hepatic echogenicity is diffuse and easy to appreciate by comparison with the lower echogenicity of the kidney or spleen.

The hepato-renal contrast is an ultrasound index for quantification the liver steatosis[37,38]. Normal liver exhibits an echostructure similar to that of renal parenchyma. In fatty liver, the increased hepatic echogenicity creates hepato-renal contrast. Webb *et al*[38] assessed the severity of liver steatosis in a study of 93 patients with positive histol-

ogy for chronic liver disease, according to the discrepancy in ultrasonographic liver-kidney densities. They reported that the hepato-renal index could quantify the severity of liver steatosis to a lower limit of 5%.

A simple parameter, noninvasive and easy to perform, is spleen longitudinal diameter. As the study of Tarantino *et al*[17] recently showed, spleen diameter could differentiate between NAFLD and NASH better than both IL-6 and vascular endothelial growth factor, with values greater than 116 mm predicting NASH[17].

Another technique that might be helpful in the diagnosis of steatosis is Doppler ultrasound. NAFLD is associated with hepatic parenchyma perfusion abnormalities. Several parameters have been described that reflect altered hepatic hemodynamics, among them, the most important is the hepatic vein Doppler pattern[3]. Recently, a new parameter was used in NAFLD evaluation: Doppler perfusion index (DPI), a ratio between hepatic arterial blood flow and total liver blood flow. DPI has been used in the detection of overt liver metastatic disease[39]. In a small trial, Dugoni *et al*[40] reported that DPI was highly predictive of fatty liver in patients with NAFLD. Larger studies are required to evaluate the role of DPI in the diagnosis of NAFLD.

The sensitivity of ultrasonography in detecting steatosis varies between 60% and 94%[18], and also varies depending on steatosis degree. Sensitivity is very low when the degree of steatosis is less than 30%[41]. Another difficulty consists of the impossibility of identifying the inflammatory changes of the hepatic parenchyma and to differentiate simple steatosis from steatohepatitis. It is also difficult to differentiate steatosis from liver fibrosis, because both of them have similar appearance on ultrasound[18]. This limitation was overcome by a superior technology, contrast-enhanced ultrasonography. Lim *et al*[42] studied the role of hepatic vein transit times (HVTT) using a microbubble contrast agent as a tracer and reported that HVTT can predict disease severity in patients with hepatitis C. Moreover, Iijima *et al*[43] evaluated the utility of contrast ultrasound with levovist for the diagnosis of NASH. The signal intensity from regions of interest on the contrast images was measured and estimated using time intensity curves. They found a statistically significant decrease of signal intensity in NASH, when compared with NAFLD, due to reduced uptake of levovist mediated by cell injury. Because this method has only been applied in small trials, larger studies are needed to establishing the role of contrast ultrasonography in the diagnosis of NASH in clinical practice.

The sensitivity of ultrasonography decreases in morbid obesity, because the ultrasonographic examination is difficult to perform in such circumstances[3]. Ultrasonography is inexpensive, simple, easily reproducible, and can be used repetitively to assess steatosis changes over the time, in conjunction with ALT fluctuations and BMI variation. The specificity of the method in detecting fatty infiltration of the liver is high, around 90%[18] (Table 2).

Computed tomography and magnetic resonance imaging are other alternatives, but their use is limited because they are expensive and the information they provide is limited. Compared with ultrasound, CT scans and MRI are superior when the fat deposition is focal[18], otherwise, abdominal ultrasound is more sensitive in diagnosing fatty liver disease[18,44,45] (Table 2).

Table 2 Noninvasive diagnosis of non-alcoholic fatty liver disease (adapted from Lewis *et al*[45]) (%)

Imaging	Sensitivity	Specificity	PPV	NPV
Ultrasound	91-100	93-100	62-89	94
Ultrasound (levovist)	100	95-100	N/A	N/A
Ultrasound (elasticity)	91	84	47	97
CT	93	N/A	76	N/A
MRI	N/A	N/A	N/A	N/A
MR (spectroscopy)	N/A	N/A	N/A	N/A
MR (elastography)	85	86	73	94

N/A: Not available; PPV: Positive predictive value; NPV: Negative predictive value; MRI: Magnetic resonance imaging; MR: Magnetic resonance; CT: Computed tomography.

CT scan technology can be also used to evaluate thickened abdominal subcutaneous adipose tissue and to measure the liver fat[46]. Non enhanced CT can identify steatosis using changes in signal intensity. The density of the liver, as visualized by CT, decreases as the severity of steatosis increases. CT can also visualize splenomegaly in the presence of portal hypertension, which is suggestive for advanced fibrosis in patients with NAFLD. CT allows grading of steatosis, by calculating the liver-to-spleen attenuation ratio[47]. Noncontrast CT is preferred for detecting steatosis because the images appear enhanced[48]. Focal fatty lesions can be identified by dual-energy CT scans. The limitations of CT consist of the difficulty to identify intermediate stages of fibrosis and its use in follow-up purposes, owing to the radiation exposure.

Magnetic resonance imaging (MRI) provides an accurate and rapid assessment of hepatic steatosis to a lower limit of 3%[49]. Phase-contrast imaging correlates with the quantitative assessment of fatty infiltration across the entire range of liver diseases. Loss of intensity on T1-weighted images can be useful in identifying focal fat deposition[49].

A new MRI technique, proton magnetic resonance spectroscopy (MRS), measures the fat proton fraction and hepatic triglyceride levels (HTGC). HTGC > 5% is the diagnostic level of hepatic steatosis[50,51]. MRS characterizes metabolic processes involved in cellular regeneration, and thus it can evaluate the disease severity in NASH. An increased ATP/phosphate ratio might be a signal for progression to an advanced stage of fibrosis in NASH. MRS is probably more accurate than previous imaging procedures for the diagnosis of NAFLD but it needs *in vivo* human validation.

Multi-echo magnetic resonance (MR) imaging, acquired at in-phase and out-of-phase echo times, allows simultaneous fat content and T2 quantification. This technique could be used to determine the fat-to-water ratio and the T2 values[52].

None of these imaging techniques is able to distinguish between liver steatosis and steatohepatitis; thus liver biopsy is required for definitive assessment of the hepatic disorder[3].

Unfortunately, the new imaging procedures, magnetic resonance spectroscopy, and contrast enhanced ultrasound cannot, as yet, be used routinely.

Table 3 Serological markers for nonalcoholic steatohepatitis and fibrosis

Serological markers	Advantage	Disadvantage
C reactive protein[53]	Independent risk factor for progression of NAFLD	Lack of specificity for NASH
Plasma pentraxin 3[54]	Can differentiate between NASH and non progressive NAFLD Fibrosis marker in NAFLD	Lack of specificity for NASH
Hyaluronic acid[34]	Identify fibrosis at a cut-off value of 45 ng/mL	Cannot differentiate NASH from simple steatosis
Tissue inhibitor of metalloproteinases[34]	Fibrosis marker	Cannot differentiate NASH from simple steatosis
Cytokeratin 18[3]	Marker of hepatocyte apoptosis Independent predictor of NASH and severity of disease	Limited utility in clinical practice
Polypeptide specific antigen[57]	Marker in differentiating NASH from pure fatty liver	Marker for various cancers
Endothelin 1[59]	Can differentiate NASH from simple steatosis	Lack of specificity for NASH

NAFLD: Non-alcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

BIOMARKERS FOR ASSESSMENT OF STEATOHEPATITIS AND FIBROSIS

Over time, several biological markers have been studied for evaluating the extent of steatosis, the presence of necroinflammation, and the development of fibrosis to avoid performing liver biopsy, an invasive procedure that still represents the gold standard of diagnosis. The most important parameter to be identified through non-invasive methods is inflammation, as it plays a central role in NAFLD progression.

Several biomarkers of inflammation were extensively studied in relation to fatty liver disease. The C reactive protein (CRP) is an acute-phase reactant produced by the liver and has an increased serum concentration in a variety of inflammatory conditions. The assessment of plasma levels of CRP proved to be useful in differentiating between simple steatosis and NASH. Moreover, it seems that high concentrations of high-sensitivity CRP are associated with extensive liver fibrosis in NASH[53].

Plasma pentraxin 3 (PTX3) is a novel marker that seems to be promising in distinguishing between NASH and non-NASH patients, and also in assessing the severity of fibrosis[54]. Plasma pentraxin 3 is an acute-phase reactant and together with CRP is a member of the pentraxin family of proteins[54]. The PTX 3 level is increased in NASH, but also in other diseases, such as vasculites, cardiovascular, and inflammatory conditions[54].

Another biomarker with a significant role in the fatty liver is IL-6. IL-6 is a chemokine that rises in NAFLD, and it is synthesized by hepatocytes and by immune cells, endothelial cells, and adipocytes[12,55]. Plasma levels of IL-6 vary in proportional with the hepatic concentration and indicate inflammatory activity and the degree of fibrosis[55].

TNF-α has long been recognized for its proinflammatory properties, and its role in NASH progression is clearly established, as well as in other inflammatory diseases. TNF-α is highly expressed in NASH and it has been shown that anti-TNF therapy with pentoxifylline is associated with improvement of liver histology and normalization of aminotransferases[56].

Cytokeratin 18 is a relatively new marker that derives from the caspase-3 pathway; however, to date, it has limited utility in clinical practice and is used only for research purposes. Cytokeratin-18 represents a marker of hepatocyte apoptosis, and its value as a potential biomarker for NASH is based on the observation that apoptosis is prominent in NASH and absent in simple steatosis[3].

Tarantino *et al*[57] found that the polypeptide specific antigen, a protein released during apoptosis, is an important marker of fibrosis, and is more accurate than ALT levels. Tissue polypeptide specific antigen seems to have a clinical utility in the follow-up of obese patients with NASH, because a significant decrease in serum concentration of this marker was associated with weight loss[58].

Oxidative stress has been documented to play a part in NASH pathogenesis, and several parameters have been assessed in different studies: glutathione peroxidase activity, superoxide dismutase activity, and vitamin E levels[3]. None of these markers seemed to have a significant value in evaluating the histological picture of NASH[3]. The clinical usefulness of these biomarkers is yet not established, and their accuracy in noninvasive assessment of steatohepatitis is under debate.

Fibrosis assessment is crucial in NASH because it represents an advanced stage of liver injury. Several studies evaluated certain matrix components, such as transforming growth factor β, hyaluronic acid, tissue inhibitors of metalloproteinases, and others[33]; however, none of them have entered routine use. Endothelin-1 is another mediator of fibrosis in NASH, with an established correlation between serum levels and the degree of fibrosis[59].

Serological markers for NASH and fibrosis are shown in Table 3.

DIAGNOSTIC PANELS FOR ASSESSMENT OF STEATOSIS, STEATOHEPATITIS AND FIBROSIS

Noninvasive panels of serological markers have been developed to evaluate the presence of steatosis and hepatic necroinflammation to avoid liver biopsy. Avoiding liver biopsy is desirable because it has certain disadvantages: it is an invasive procedure, is prone to sampling errors, and suffers from inter-observer variability[60]. The NASH-test imagined by BioPredictive was validated for the assessment of

steatohepatitis in patients without significant alcohol consumption and takes into account the following parameters: total bilirubin, GGT, α2-macroglobulin, apolipoprotein A1, haptoglobin and ALT, and is adjusted for age and gender plus[61] weight, height, AST, serum glucose, triglycerides, cholesterol and SteatoTest. The NASH test should be performed only if the SteatoTest is positive. The SteatoTest is a quantitative test that estimates liver steatosis, particularly in cases of associated metabolic syndrome[62]. The NASH test is a variation of the SteatoTest-ActiTest for the differentiation of steatosis from NASH. The Acti Test was designed for staging necroinflammation in viral hepatitis C and B[61]. Performing these biomarker tests should reduce the need for liver biopsy[63].

Serological markers for fibrosis assessment are frequently used in Europe, in contrast with the United States where liver biopsy is preferred. Different tests have been used for evaluating fibrosis, such as the AST/ALT ratio and the APRI test, which assesses platelets and AST levels[64]. At the moment, the most commonly used are the FibroTest (BioPredictive) in Europe, and FibroSpect and FibroSure in the United States[64]. FibroTest was first developed for patients with viral hepatitis C, and was then extended for NAFLD[33]. The advantages over liver biopsy are: entire examination of the liver and lack of risks due to the noninvasive procedure. FibroSpect evaluates liver fibrosis by analyzing the following markers: hyaluronic acid, tissue-inhibited matrix metalloproteinase inhibitor-1, and α-2 macroglobulin[64]. FibroTest takes into account GGT, haptoglobin, bilirubin, apolipoprotein A, and α-2-macroglobulin. The most important deficiency of these types of tests is their inability to distinguish between mild and moderate fibrosis, knowing that early detection of fibrosis is valuable for preventing disease progression[64]. The utility of these tests is limited in cases with advanced fibrosis.

Fibroscan

Fibroscan, or transient elastography, is a noninvasive method that evaluates liver stiffness using pulse-echo ultrasound[33,64]. Transient elastography measures liver stiffness in a painless and reproducible manner. It has several advantages over liver biopsy: it is noninvasive, evaluates a larger part of the liver, and seems to be more sensitive than serological markers[64]. The main weakness of Fibroscan is interference by steatosis with the wave velocity, as liver stiffness due to fibrosis might be counterbalanced by the presence of fatty infiltration[33]. Some authors state a positive correlation between liver stiffness assessed by Fibroscan and the degree of fibrosis in NAFLD[65,66]. When liver elasticity is used for fibrosis measurement in NAFLD, it is important to take into account that fatty liver can make the liver less stiff and therefore the reference ranges might be different. Fibroscan might also be unreliable in obese people because of technical reasons[67].

Acoustic radiation force impulse (ARFI) sonoelastography has recently been proposed as an alternative method to Fibroscan to assess liver elasticity. This alternative technique utilizes acoustic waves to interrogate the mechanical stiffness properties of the liver. One advantage of ARFI imaging is that it is integrated into a conventional ultrasonography (US) system and can thus be performed during standard US examinations of the liver, which are routinely performed in patients with chronic liver disease. Preliminary results indicate that ARFI imaging technology can be applied for the diagnosis of significant liver fibrosis[68,69]. The role of ARFI elastography for the diagnosis of NAFLD has not yet been established.

Another technique used for detecting moderate to severe hepatic fibrosis in obese individuals with NAFLD is magnetic resonance elastography. It has a higher diagnostic accuracy in fibrosis staging that is not related to BMI[70]. Further studies are needed to clearly define the role of liver elastography in patients with fatty liver disease.

Total overnight salivary caffeine assessment test

An interesting idea concerning the assessment of liver function in chronic liver diseases was elaborated by a working group conducted by Tarantino *et al*[71]. Systemic caffeine clearance, evidenced by measuring salivary caffeine concentration can be used as a hepatic function test in compensated cirrhosis. The total overnight salivary caffeine assessment is a reliable test for evaluating liver function and it can also differentiate between cirrhosis type, such as viral and cryptogenic (likely metabolic) cirrhosis.

Dynamic breath tests

Dynamic breath tests can detect specific alterations in different metabolic pathways. Braun *et al*[72] combined two tests to assess the extent of hepatic injury in patients with NAFLD: the ^{13}C-methacetin breath test (MBT) and the ^{13}C-octanoate breath test (OBT), which evaluate cytochrome P450 activity and mitochondrial dysfunction. Both mechanisms increase oxidative stress, which is clearly implicated in NASH pathogenesis. The noninvasive OBT reliably distinguish between fatty liver and NASH, and the MBT can predict the extent of liver fibrosis.

Additional studies are required to establish the role of these tests as an alternative to liver biopsy in the diagnosis and follow-up of hepatic injury in patients with NAFLD.

CONCLUSION

Currently, the standard procedure for evaluating the degree of necroinflammation and fibrosis, and for quantifying hepatic steatosis remains liver biopsy. However, this is an invasive procedure with unavoidable risks and limitations. Moreover, in most cases of NAFLD, the results of liver biopsy are not relevant to the choice of treatment, which remains that of metabolic syndrome. Hence the need for noninvasive strategies to cover the whole spectrum of NAFLD. Noninvasive investigations, such as various biomarkers, fibrosis scoring panels, and imaging techniques, offer considerable promise in their ability to detect steatosis and to stage liver fibrosis. Further testing and validation are needed for these noninvasive procedures to refine their role of clinical practice and supplant the need for liver biopsy in NAFLD.

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