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DIAGNOSTIC ADVANCES

Nonalcoholic fatty liver disease: Diagnostic biomarkers

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Abstract

Nonalcoholic fatty liver disease is a common medical condition worldwide and its prevalence has increased notably in the past few years due to the increases in prevalence of obesity and metabolic syndrome. However, diagnosis of this disease is still a matter of debate because of disease variations and pathophysiologic alterations. Specific single markers have gained considerable attention recently, among them markers related to hepatic pathophysiology, inflammation, adipocytokines and so forth. But, it seems that no single marker is sufficient for diagnosis and staging of the disease, and applying a panel including different types of tests may be more useful.

Key words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Liver fibrosis; Cirrhosis

Core tip: Nonalcoholic fatty liver disease is a pandemic disease in both developed and developing countries. There is emerging scientific evidence in this field that needs to be classified and summarized to make conceptual maps for researchers as well as practitioners. This article aimed to cover diagnostic markers in this disease, considering limitations and applications.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most common chronic hepatic diseases in many developed countries. It is also a serious health problem all over the world[1]. This disease includes a broad range of hepatic disorders from simple fat accumulation in hepatic cells (simple steatosis) to hepatic tissue

inflammation and fibrosis (steatohepatitis) and finally cirrhosis and even hepatocellular carcinoma[2-6]. The prevalence of this disease has considerably increased in the past years, and different studies have reported 2-fold increase in its prevalence within 10 years in many countries[7-9].

Diagnosis of NAFLD is of special importance because of the odds of its progressing to more critical stages. Furthermore, in cases of diagnosis at early stages, it is possible to prevent disease aggravation by applying simple approaches such as increasing physical activity and diet modification. Even in more advanced stages of the disease, such as non-alcoholic steatohepatitis (NASH), it is still important to diagnose it as early as possible due to its potential for progressing to cirrhosis[10]. In patients suspected of having cirrhosis, it is necessary to take other diagnostic measures into consideration, such as studying complications of portal hypertension (*e.g.,* screening for gasteroesophageal varicose veins) and examining the risk of hepatocellular carcinoma development[11-14]. So, it is important to be able to differentiate between simple steatosis and steatohepatitis as the beginning step of the progression towards severe and more progressive stages of the disease, such as fibrosis and cirrhosis[15,16].

At present, liver biopsy is the gold standard for diagnosing nonalcoholic fatty liver, but this method is not only invasive and expensive but also has the important limitations of pain, reluctance of patients, risk of severe complications and being subject to sampling error. All these challenges have increased motivation for finding/applying noninvasive methods for diagnosis of the different stages of NAFLD[17]. In this study, we provide a brief review of the individual main indices invented for the diagnosis and prediction of different stages of NAFLD.

HEPATIC MARKERS

More than 60 transamination reactions have been recognized in liver, among which only the transaminases of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are of clinical value. Both of these markers, but especially ALT, are commonly used in clinical medicine as indicators of hepatic damage[18]. In many studies on patients suffering from NAFLD, increased levels of aminotransferases along with diabetes have been considered as independent predictors of moderate to severe fibrosis[19] in patients with fatty liver who are at risk of progression to advanced fibrosis and only these two variables have been shown to have a significant association with steatohepatitis[20].

ALT

In many people, the asymptomatic increase of transaminases level, particularly ALT, has been observed, and the prevalence of this type of case (asymptomatic increase of ALT) has been reported differently in different populations (7.3% in the United States), 15.24% in Spain, and 21.7% in Scotland)[18,21], but it generally seems that it has a prevalence of 4% to 21.7%[18]. Of note, almost one-third of the people who have shown increased level of serum transaminases in their first test have demonstrated normal level of enzyme in the second one[18].

High waist circumference, high body mass index (BMI), male sex, alcohol consumption and young age are considered as strong predictors for rising ALT level[21]. Moreover, ALT level has been shown to have a positive association with the number of signs and symptoms of metabolic syndrome present[22]. In addition, serum ALT level has been shown to have a positive correlation with serum triglyceride, serum dehydroepiandrosterone sulfate (DHEAS), plasma fasting glucose and BMI, and a negative correlation with hemoglobin (Hb), serum high-density lipoprotein and age[23].

In some studies, NASH has been reported as the most prevalent cause of constant increases in serum ALT in asymptomatic blood donors[24] and it has been demonstrated that 32% of people who had ultrasound examination have been diagnosed with steatohepatitis accompanying asymptomatic increases of transaminases level[18]. In another study, intense fibrosis was shown to be independently predictable by ALT and two other indices (serum ferritin and diabetes)[25]. Other studies have also indicated a relationship between presence of steatohepatitis and increased level of ALT[26], and even ALT has been considered as a part of some steatohepatitis diagnostic panels, like the BAAT score[27], FIB4 index[28], FibroTest[29], FibroMeter[30], NashTest[29] and NFS[30].

In contrast, some studies have demonstrated that some patients suffering from NAFLD have normal serum level of ALT. In a study on a United States' population (the Dallas Heart Study), 79% of the adults with fatty liver had normal aminotransferase level[31], which was the same as the 55% of the Italian adults suffering from this disease in another study (the Dionysos Study)[32]. In a study of 2287 individuals from different tribes, almost one-third of the population had hepatic steatosis, among whom 79% showed normal ALT level[33]. In another study, normal serum transaminases level was reported in 46% of patients with steatohepatitis[34]. Of interest, a research study into the risk of NAFLD development in cases of normal serum levels of ALT (*i.e.,* lower than 35 U/L) indicated that the rise in serum ALT concentration - even within the normal range - was an independent predictor for NAFLD development[35]. In a study on diabetic patients, it was also shown that high-normal ALT could increase the risk of nonalcoholic fatty liver by 2.7-times[36].

There is no consensus on cutoff point and maximum normal concentration of ALT, and a broad value range, between 26 and 66 IU/L, has been suggested in different studies[37]. Some studies have shown that risk of NAFLD increases as ALT rises to more than 19 and 30 in women and men, respectively. Accordingly, these values have been suggested as maximum probable normal values in

these groups[38]. In a community-based study of 1346 Japanese patients with diagnostic criteria of NAFLD, the cutoff value of ALT for diagnosis of this disease was estimated to be 25 U/L in men and 17 U/L in women[39]. For the BAAT score, a normal range of ALT is considered between 1 and 19[27]. In another study that considered 19 and 30 (for women and men respectively) as the cutoff values for screening NASH among NAFLD patients, 99% and 8% sensitivity and specificity respectively were shown, while 40 U/L - which is in use in medicine - had 86% sensitivity and 32% specificity[26].

Thus, some researchers have rejected considering a reduction of ALT level (within reference range) as a tool for increasing its sensitivity because it leads to the adverse effect of labeling large numbers of asymptomatic individuals as patients[40]. Nevertheless, while some researchers believe that none of these values has enough sensitivity and specificity to support introduction of ALT as a reliable screening test for diagnosing steatohepatitis among patients with fatty liver[26,41]. Some other researches have suggested this enzyme as the best individual marker for detecting fat infiltration in the liver[42].

Riquelme *et al*[43] showed that, in a Hispanic population, ALT greater than 14 had an odds ratio (OR) equal to 13.95 for fatty liver. Moreover, although some studies have mentioned that this enzyme cannot be considered as an index for prediction/diagnosis of steatosis or fibrosis[44], in a study conducted on patients with type 2 diabetes, ALT was the only variable independently related to steatohepatitis in these patients[45]. In another study, ALT was found to be considerably higher in patients with steatohepatitis, even when within normal ranges[45]. And, a study on obese patients showed that elevated ALT level had a significant correlation with incidence of steatohepatitis and fibrosis[34,46]. In contrast, a study conducted on 73 untreated patients - to investigate the relationship between changes in hepatic enzymes and histological liver changes in NAFLD - indicated absence of a clear relationship between the pattern of ALT levels and changes in steatosis, inflammation, hepatocyte ballooning or degree of fibrosis over time. Results of this study also demonstrated the level of hepatic enzymes as an insensitive tool for following histological changes of liver among patients with NAFLD[47]. Regarding more advance stages of the disease, Kaneda *et al*[48] showed ALT level to be significantly different in patients with cirrhosis, compared to patients with earlier stages (50 IU/L *vs* 87 IU/L).

AST

AST is another hepatic transaminase that plays a role in diagnosis of steatohepatitis. Up to 3.6% of people in the United States have asymptomatic increase in AST[21], and it seems that this hepatic index is related to metabolic syndrome and BMI[49]. In Asian studies, AST is considered as an independent marker for severity of hepatic fibrosis if it is at least twice as much as the maximum normal value[50]. This marker has been used in different diagnostic panels, such as the FibroTest[12,29,51], NashTest[52], NAFLD Fibrosis Score (NFS)[53,54], FibroMeter[55], FIB4 index[28] and other different models[56,57]. In addition, two common tests of AST to ALT ratio and the AST to platelet ratio index (APRI) have been generated using this enzyme.

The AST to ALT ratio, which is frequently used in medicine, is known as an independent index for predicting presence of advanced hepatic fibrosis and has been used as a part of different panels, such as the NAFLD Fibrosis Score[26], ScoreBARD[58] and other panels[28,41,57]. The APRI is another non-invasive index for studying hepatic fibrosis in patients with NAFLD and it has been reported as preferred over the AST to ALT ratio for predicting advanced hepatic fibrosis[59].

Alkaline phosphatase

Higher level of alkaline phosphatase (ALKP) can also be considered as a marker relating to hepatic fibrosis in patients with steatohepatitis. In a study conducted on NASH patients to investigate the association between serum levels of ALKP with the degree of hepatic fibrosis, it was shown that serum level of this enzyme was significantly higher in patients suffering from NASH as compared to those without it[60]. Another study represented ALKP to be significantly higher in NASH patients, even when within normal range[45]. In addition, the increased level of ALKP has been reported as an independent predictor for hepatic diseases-related death[61].

Albumin

In different studies albumin has been identified as a factor related to steatohepatitis, septal and intensive fibrosis[27,48,62] and cirrhosis[48] and as an independent predictor of hepatic-related mortality[61,63]. In a study on 73 untreated NAFLD patients, a significant decrease was reported in serum albumin level of patients during 2-year follow-up (from 0.5 ± 4.3 at the beginning of the study to 0.4 ± 4.2 after 24 mo)[47]. In another study, serum level of this protein showed OR of 0.049 (95%CI: 0.003-0.879) and of 0.057 (95%CI: 0.007-0.477) for prediction of steatohepatitis and severe fibrosis, respectively[62].

In an investigation by Suzuki *et al*[64], serum albumin was reported significantly lower in patients with intensive hepatic fibrosis (stage 3-4) compared to those in earlier stages (4.1 ± 0.09 *vs* 4.4 ± 0.05). In another study, serum level of albumin was reported as 4.4 g/dL (range: 3.5-5.4 g/dL) in patients with mild fibrosis and 4.2 g/dL (range: 4.6-2.6 g/dL) in patients with intensive fibrosis, and the difference was statistically significant[48]. In the same study, the serum level of this protein showed a significant difference in patients with and without cirrhosis (4 g/dL *vs* 4.4 g/dL)[48]. However, in a study by Sumida *et al*[65], although concentration of serum albumin showed diminishment when the

intensity of NAFLD increased, this decreasing trend was not statistically significant (4.51 g/dL in simple steatosis, 4.37 g/dL in mild steatosis and 4.29 g/dL in advanced steatosis).

INFLAMMATORY MARKERS

Platelets

Thrombocytopenia is a common finding in advanced stages of all chronic hepatic diseases, and platelet count is clinically important to predict status of hepatic fibrosis in patients with chronic hepatic diseases[66]. In NAFLD, platelets are important in terms of absolute number as well as volume. In different studies, reduction of platelets to fewer than 160000 has been considered as an independent marker for severity of hepatic fibrosis[17,50].

In a study by Suzuki *et al*[64], platelet count was reportedly significantly lower in patients with intensive hepatic fibrosis (stage 3-4), compared to patients in earlier stages (180 ± 17 10^3/mm^3 *vs* 247 ± 10 10^3/mm^3). The result of this study has been confirmed by other studies[48]. In a study in 2006, when the cutoff point of 16 × 10^4/μL was used, platelet count was shown to have 100% sensitivity, 95% specificity, 76% positive predictive value (PPV) and 100% negative predictive value (NPV) for diagnosis of cirrhosis[48]. In this study, platelet count was determined as an independent predictor among different markers and it was shown that this index significantly decreased in cases of cirrhosis (from 241 × 10^3/μL to 130 × 10^3/μL)[48]. In addition, in another study conducted on NAFLD patients from 9 hepatology centers in Japan, it was shown that platelet count linearly decreased with the increase of histological intensity of hepatic fibrosis. The cutoff value of 19.2 × 10^4 was introduced and shown to provide a sensitivity of 62.7% and a specificity of 76.3% for diagnosis of the 3rd stage of hepatic fibrosis, while 15.3 × 10^4 was selected as the optimal cutoff value for diagnosis of the last stage of this disease (sensitivity: 80.5%, specificity: 88.8%)[67].

Mean platelet volume (MPV) is considered as an indicator of platelet function[68]. In 2010, Ozhan *et al*[69] showed that patients with NAFLD have higher MPV than individuals without it (10.43 ± 1.14 *vs* 9.09 ± 1.25). This study also showed that MPV had a positive correlation with AST and ALT levels, and a negative correlation with platelet count. These results have been confirmed in another study conducted on obese patients which showed a significant association between NASH and MPV, and which indicated an increase in the prevalence of NASH by increasing the values of MPV after adjustment for all the confounding variables[70].

In contrast, another study with 60 NAFLD cases and 54 healthy controls, the MPV did not show any difference between the two groups. This study also concluded that in the absence of other MPV metabolic risk factors, MPV does not play a role in the mechanism of increasing the risk of cardiovascular diseases in patients with fatty liver[71]. Moreover, in another study in 2012, which was conducted to investigate the relationship between MPV index and NAFLD, although MPV was increased in patients with NAFLD, no association was detected between degree of steatosis, lobular hepatitis, hepatocellular ballooning, NAFLD activity score and fibrosis with the values of MPV. In this study, it did not show a correlation with increase of resistance to insulin[68].

Considering the novelty of this hypothesis (significant association between MPV and NAFLD) and remembering the scarcity of the studies conducted to examine this hypothesis so far, it seems that there is still need for more studies to make a clear conclusion. Furthermore, considering that platelets usually decrease in stage 4, it can be probably concluded that a decrease in platelets usually indicates the occurrence of more advanced stages of fibrosis, which is usually equivalent to cirrhosis[70]. Since platelet count is a simple, cost-effective and accurate method, it can be considered as a suitable biomarker for diagnosis of fibrosis severity in these patients[72].

C-reactive protein

C-reactive protein (CRP) is an acute phase reactant, which has many applications in clinic[73]. It seems that serum concentration of CRP is a strong index for predicting the incidence of NAFLD[74] and some studies have introduced increased serum level of CRP as an independent risk factor for development of NAFLD[74,75]. In addition to routine measurement of CRP, another method has been invented to measure values of high-sensitivity CRP (hs-CRP). This method promises to make detection of even low degrees of inflammation possible[76]. In some studies, hs-CRP has been called a diagnostic tool, which not only can be effective in differentiation of steatohepatitis but also can specify the severity degree of hepatic fibrosis in patients with NASH and is probably able to differentiate between advanced and mild fibrosis among these patients[65,76]. This ability has been mentioned to persist even after adjusting for the effect of different confounding variables, such of age, sex, diabetes, dyslipidemia, BMI, subcutaneous fat and intra-abdominal visceral fat[76]. Another research study has shown that hs-CRP level was higher in patients with more severe grades of steatohepatitis (grades 2 and 3), rather than in patients with mild or simple grades of NASH[77].

Although several studies have shown a relationship between NAFLD and serum concentration of hs-CRP, results of some studies are in contrast to this hypothesis[74]. It was concluded in one study that hs-CRP could be an index for determining steatosis in obese patients, but there is no association between this marker and NASH[78]. In parallel, a cohort study could not find any relationship between hs-CRP level and severity of hepatic steatosis. The result of this study indicated that hs-CRP is not helpful in predicting histological intensity of NAFLD[73]. Of note, some investigations

have also demonstrated increased serum levels of hs-CRP in disorders related to NAFLD, such as obesity, insulin resistance and manifestations of metabolic syndrome[73,78-82]; although, there is doubt about the presence of a causal relationship between this marker and metabolic syndrome[79].

CRP is mostly produced in liver, but it seems that it is also produced in the adipose tissue[80]. Furthermore, adipose tissue can act as an endocrine organ and secretes some inflammatory cytokines, like interleukin (IL)-6, leading to stimulation of the liver for CRP production[83]. So, generally, it seems that obesity is one of the strongest determinants of serum level of CRP. Of interest, serum level of CRP is higher in women than in men[83-85], and the results of one study have shown that an increase in serum level of hs-CRP is significantly associated with intensity of NAFLD, only in women[74]. One explanation for this could be related to the sex difference in correlation between CRP and obesity, which in turn can be justified by the difference between the two sexes in terms of amount and pattern of adipose tissue distribution[83].

One of the disadvantages of this index is the effect of different factors, such as race, age, sex, smoking or alcohol consumption, on its serum concentration[74,86,87]. On the other hand, it seems that an accurate and accepted cutoff point has not yet been determined for this marker. Median CRP value of about 1 has been introduced for diagnosis of steatohepatitis, while the suggested cutoff value for hs-CRP for diagnosis of metabolic syndrome, NASH and prediction of the risk of cardiovascular complications is 0.65 mg/L[88].

In sum, considering most studies conducted in this field, it seems that this index can be regarded as a promising biomarker for screening steatohepatitis in the future[77].

Iron (ferritin)

Iron is considered as an element that reacts to oxygen radicals. High rates of blood ferritin and increased iron accumulation in liver have been reported in steatohepatitis, which can be attributed to systemic inflammation, increase in iron storage, or both[89]. Ferritin level usually increases in 20%-50% and transferrin saturation increases in 5%-10% of NAFLD patients[90]. One study showed that elevation of serum ferritin level by 1.5-times as much as the maximum normal rate was related to accumulation of iron in liver, diagnosis of steatohepatitis and worsening of histological activity of this disease. It has been introduced as an independent index for diagnosis of advanced hepatic fibrosis among patients with fatty liver, which can probably be applied as a useful index for identifying the patients who are susceptible to steatohepatitis and fibrosis[89].

In the NAFIC scoring method, serum ferritin levels of equal or more than 200 ng/mL and 300 ng/mL in women and men, respectively, have been used as an independent variable for diagnosis of NASH[91]. Of note, although the study by Sumida *et al*[91] showed an increase in concentration of ferritin by increasing the severity of NAFLD, the difference was not statistically significant (179 ng/mL in simple steatosis, 241 ng/mL in mild steatohepatitis and 278.1 ng/mL in advanced steatohepatitis). On the other hand, some researchers believe that hemocystein level in serum may be able to independently predict steatohepatitis and could be applied as another noninvasive marker for evaluating NAFLD. Hemocystein level of serum in NAFLD is not different from that in healthy people, while it is considerably decreased in patients with steatohepatitis[92].

Malondialdehyde

In the two-hit theory - one of the most acceptable theories to justify the progression of NAFLD to NASH - an oxidative stress, which may lead to lipid peroxidation, is considered as the most probable mechanism for the second hit. During the process of lipid peroxidation, a wide range of pre-inflammatory and fibrogen products are produced as by-products, which result in progression of the disease. One of these by-products is Malondialdehyde (MDA)[93]. MDA can stimulate hepatic stellate cells and result in fibrosis by producing collagen[94].

In a study from 2010, MDA with a cutoff point of 11 had sensitivity of 60%, specificity of 92%, PPV of 81%, NPV of 81% and positive likelihood ratio (+LR) of 7.9 for predicting the presence of NASH[65]. Another study which investigated the relationship between MDA level as index for oxidative stress, antioxidant vitamins A and alpha-tocopherol with presence of steatohepatitis showed that level of vitamin A and MDA increased in simple steatosis and steatohepatitis and alpha-tocopherol level significantly decreased in patients with steatosis and steatohepatitis compared with healthy people. In this study, although there was significant difference between patients with steatosis and steatohepatitis, the authors concluded that none of these indices had relationship with histopathologic severity of the disease[93].

Plasma pentraxin 3

Pentraxin is a family of proteins divided into two short and long classes, based on the length of their structure. CRP and serum amyloid P are two short parts of this family. Plasma pentraxin 3 (PTX3) is one of the long proteins of this family, and it is significantly higher in patients with steatohepatitis than those without. Serum concentration of this protein is higher in patients suffering from more advanced stages of NAFLD and higher values of this protein are correlated with higher stages of the hepatic fibrosis. So, it seems that serum PTX3 level could be used as a marker for diagnosis of the severity of hepatic fibrosis, in addition to differentiating between steatohepatitis and simple steatosis[95].

Adipocytokines

Adipocytokines are a large family of proteins produced

and secreted by adipose tissue and which have close relationship with the inflammation process[87]. Adipokines and cytokines play a major role in regulation and orchestration of inflammatory processes all over the body and play an important role in insulin resistance pathogenesis and NAFLD through complex and mutual paracrine and endocrine mechanisms. Some adipocytokines reduce insulin resistance, like adiponectin and leptin, while others lead to increased insulin resistance, like IL-6 and tumor necrosis factor-alpha (TNF-α)[96].

Some studies have shown that the concentration of serum adipokines in human can be used as an index for diagnosis of NAFLD, especially in its advanced stages[46]; however, other studies have not been able to find a significant association between adipokines and histopathological intensity of this disease[97] and it has been concluded that adipokines cannot differentiate between benign and advanced histological stages of NAFLD[46]. It seems that there is a complex relationship between adipocytokines and pathogenesis of NAFLD[98], and the balance between proinflammatory and anti-inflammatory function of adipocytokines may play an important role in the development of this disease[99].

Adiponectin

Adiponectin is a collagen-like protein derived from adipocytes with anti-inflammatory and anti-lipogenic effects[100]. This adipokine, which is circulating abundantly in human serum, protects against excessive accumulation of fat in the liver and subsequently protects liver against inflammation and fibrosis. Serum level of adiponectin decreases in obese people and shows greater decrease in patients with steatosis and steatohepatitis[7,46,101], being higher in patients with lower degrees of steatosis[102]. Hypoadiponectinemia has been demonstrated to be a predictive factor for necro-inflammation and more severe grades of fibrosis, even after exclusion of the effect of variables such as age, BMI and waist circumference[103]. It is among the variables which have a direct relationship with steatohepatitis as compared to simple steatosis[98].

A study designed to measure liver fat, intraabdominal fat and subcutaneous fat, along with insulin resistance indices and adiponectin, showed that serum adiponectin had a reverse relationship with hepatic fat content[9]. Hypoadiponectinemia is accompanied by increased risk of cardiovascular diseases and it seems to be a key factor in metabolic syndrome[102], although some studies have shown that hypoadiponectinemia in steatohepatitis is independent from insulin resistance[73,100]. It seems that hypoadiponectinemia is a primary finding in steatohepatitis, which is identifiable from a very long time before the appearance of diabetes or emergence of central obesity and which has a correlation with histological intensity of hepatic damage.

The serum level of adiponectin was shown to be significantly lower in patients with steatohepatitis than in a control group (344 ± 5.476 *vs* 836 ± 11.548), while no difference was detected between the two groups in terms of other cytokines[103]. In another study, the serum level of adiponectin was found to be significantly lower in patients with primary stages of steatohepatitis, compared to patients with simple steatosis (3.6 mug/mL *vs* 6.0 mug/mL). In this study, adiponectin showed higher differentiation power than serum level of type Ⅳ collagen 7s and the homeostasis model assessment of insulin resistance (HOMA-IR) and it had sensitivity of 68% and specificity of 79% for predicting primary stages of steatohepatitis[104].

Considering the association between single nucleotide polymorphisms (SNPs) of the adiponectin gene and insulin resistance and increase of prevalence of type 2 diabetes, a study was conducted to determine the association between variations in this gene and NAFLD development, which indicated a positive association, especially with hepatic fibrosis[105]. Another study showed a considerable decrease in the mRNA expression of adiponectin and RⅡ receptors (adipoRⅡ) in the liver of NASH patients as compared to patients with simple steatosis, which might be indicative for its pathophysiological relationship with NAFLD[106].

Adiponectin is a powerful anti-inflammatory adipocytokine for neutralizing TNF-α[99]. Lack of a relationship between circulating levels of adiponectin and its hepatic expression, shown in the study by Kaser *et al*[106] was interpreted such that hepatic expression of adiponectin was probably regulated by different factors, such as TNF-α. Owing to the importance of adiponectin as a diagnostic marker, it has been used in different noninvasive panels for diagnosis of NAFLD[104,107].

Leptin

Leptin is a 16 kD non-glycosylated protein which is usually secreted from adipocytes of white adipose tissue[102,108]. Also, low amounts of leptin has been shown to be secreted by other tissues, such as placenta, skeletal muscles, stomach fundus and culture-activated hepatic stellate cells[108]. Leptin plays the role of a peptide hormone, regulating food uptake and energy consumption of the body through central feedback mechanisms and relating eating to hypothalamus and adipose tissue mass (*i.e.,* it controls food uptake and increases energy consumption)[102,108,109]. The importance of this regulatory role is such that recombinant leptin has been studied for treatment of all prevalent types of obesity in different clinical trials[110-113].

Some researchers believe that serum leptin level is associated with NAFLD. It has been shown that increased serum leptin level in patients with steatohepatitis has no relation with BMI of these patients, and this increase could not be easily justified by the patient's sex, obesity or type 2 diabetes[109,114]. In contrast, another study indicated a correlation between human serum level of leptin and body fat percent and BMI[115]. A study which was conducted on young adults (18-year-old to 21-year-

old students) to determine the risk factors of NAFLD in this age group showed serum level of leptin to be associated with abdominal wall fat index (AFI) (as the only independent risk factor of fatty liver in this study) in women, while it had no correlation with AFI in men[116].

Some studies have indicated a role for leptin in resistance of liver against insulin[115], and some others have connected leptin to atherosclerosis and cardiovascular diseases in obese patients[102]. However, a study designed to investigate the potential association between leptin with insulin resistance and histological changes in NAFLD patients was unsuccessful to show any association between serum level of leptin with fasting insulin level and severity of hepatic histological changes[117]. Another study conducted in 2003 showed increase in the serum level of leptin as one of the presentations of patients with steatosis and normal serum level of transaminases. In this study, a negative correlation was detected between serum leptin and serum transaminase levels, as well as progression of hepatocytes damage[118]. Another study which followed patients for 6 mo showed considerable decrease in the level of serum transaminase only in NASH patients with increased levels of serum leptin. This study concluded that leptin has a preventive effect on progression of hepatic damage in NAFLD[117].

Different studies have been conducted so far on factors relating to leptin and its role in the pathogenesis of NAFLD[119]. Some studies have shown that serum leptin level is directly associated with severity of hepatic steatosis and that increased levels of serum leptin may lead to increase of hepatic steatosis and steatohepatitis, but no association with emergence of inflammation and fibrosis was found[109,114]. Some other studies, however, have failed to show a significant association between serum level of leptin and steatosis[120] or steatohepatitis[46]. Still other studies have concluded that leptin could be used for diagnosis of hepatic fibrosis, but not for staging of this disease[121].

TNF-α

TNF-α plays roles in the progression of NAFLD through different mechanisms. TNF-α has pro-inflammatory effects and activates harmful pathogenic routes by decreasing HDL-cholesterol, increasing expression of cholesterogenic genes and suppressing cholesterol exclusion[122]. It also stimulates synthesis of hepatic fatty acids, increases serum level of triglyceride[123] and decreases sensitivity to insulin[100,124]. In addition, TNF-α can also induce apoptosis in and proliferation of hepatic cells and play a role in pathogenesis of hepatic fibrosis[124]. The importance of TNF-α in emergence of fatty liver disease (with genetic or nutritional origin) has been demonstrated in different studies, and it has been shown that neutralization of TNF-α activity leads to improvement of insulin resistance and fatty liver disease[111].

Different studies have demonstrated that plasma level of TNF-α is higher in patients with NAFLD and NASH, compared to control groups[73,98,125-128], significantly or at least borderline significantly (P = 0.052)[129]. In a study by Jarrar *et al*[98], TNF-α was introduced as the only independent predictor of fibrosis in patients with steatohepatitis. In another study conducted in 2005 to examine TNF-α as a potential noninvasive marker for studying histopathological intensity of NASH, serum level of TNF-α was higher in patients with steatohepatitis and cirrhosis than in the control group, but it did not show a significant association with histopathological severity of the disease[126]. Another confirmatory study by Hui *et al*[100] showed that TNF-α level was higher in patients with fatty liver and steatohepatitis as compared to healthy individuals, but it was not efficient for differentiating steatohepatitis from fatty liver.

A study in 2010 conducted on NAFLD children, showed that serum concentration of TNF-α and its soluble receptors were significantly higher in obese children with fatty liver than those in the control group. But, again, the abilities of this cytokine and its receptors were insufficient in differentiating between different grades of steatosis. In this study, this marker was introduced as a suitable serum marker for predicting hepatic steatosis in obese children[127]. However, another study conducted in 2005 did not find a significant difference between TNF-α level of patients with steatohepatitis as compared to the control group[103].

Of note, TNF-α usually increases in visceral obesity and NAFLD, and it has been shown that this cytokine plays a role in regulation of the body's iron homeostasis. A study in 2008 which investigated these associations showed that the highest serum concentration of this cytokine was found in NAFLD patients with iron overload. It has also been shown that phlebotomy treatment can reduce concentration of TNF-α in addition to level of ferritin and transferrin saturation and can lead to improvements in hepatic function tests. This study concluded that TNF-α plays a regulatory role for iron that leads to accumulation of ion in the liver of patients with NAFLD[125].

IL-6

IL-6 is a multifunctional cytokine which regulates immune responses, the acute phase reaction and homeostasis[124] and is secreted from different cells in body, one of which is the adipocytes[83,100]. This cytokine causes stimulation of liver and some acute phase proteins, like PTX3 and CRP, are then produced in liver in response to it[129,130]. Serum level of IL-6 has considerable correlation with insulin-resistance, like TNF-α, which leads to increased resistance to insulin[99,100]. Also, IL-6 stimulates hepatic lipogenesis related to obesity and insulin-resistance[125]. It seems that this cytokine plays a critical role in pathophysiology of different aspects of NAFLD in humans[99].

Zamora-Valdés *et al*[131] showed in 2007 that chronic alternating hypoxia resulting from obstructive

sleep apnea, as one of the risk factors for NAFLD, led to increased serum level of IL-6. IL-6 level has also been shown to be significantly different between NAFLD patients and non-afflicted people in some studies[98]. Another study indicated a considerable increase in the serum level of TNF and IL-6 as well as of their soluble receptors in steatohepatitis, compared to simple steatosis, but no correlation was found between circulating levels of these cytokines and their receptors with either the degree of disease activity or fibrosis stage[132]. However, another study did not find any increase in the hepatic mRNA transcription and expression of IL-6 in patients with steatohepatitis as compared to patients with simple steatosis[76].

STRUCTURAL MARKERS

Cytokeratin-18

Different studies have indicated the key role of apoptosis in hepatic damage occurring in advanced stages of NAFLD (steatohepatitis) and, subsequently, use of hepatocyte apoptosis markers was suggested for diagnosis and staging of this disease[133-135]. During the apoptosis process, cytokeratin-18 (CK-18) is fragmented by caspase 3[135] and hepatocytes which leads to cell death through the apoptosis process and release fragments of CK-18 into the blood stream, so that the blood level of the fragmented CK-18 is associated with the presence of hepatic fibrosis[133]. Indeed, this substance has been shown to be significantly higher in patients with NASH[120].

Plasma CK-18 fragments have been studied and evaluated in large studies[72,136,137]. In one study, for diagnosis of NASH, blood level of more than 395 U/L of cytokeratin had sensitivity, specificity, PPV and NPV values of 85.7, 99.9, 99.9 and 85.7, respectively[134]. Younossi *et al*[107] reported sensitivity, specificity, PPV and NPV of cleaved CK-18 as 63.64, 87.23, 70 and 83.7, respectively, for the cutoff value of 174.1; however, when the cutoff point was increased to 261.35, specificity and PPV increased to 97.87 and 88.9, and when the cutoff decreased to 111.6, sensitivity and NPV changed to 81.82 and 77.8, respectively. In another study, sensitivity and specificity of this index were reported to be 78% and 87%, respectively, for diagnosis of steatohepatitis[138].

In one study conducted to investigate the clinical utility of different serum markers such as CK-18, hyaluronic acid and tissue inhibitor of metalloproteinase 1 (TIMP1) for the diagnosis of steatohepatitis, CK-18 was the only biomarker which could be helpful, having PPV of 81% and NPV of 85%[139]. In addition to the cleaved form of CK-18, its intact form, and also the difference between these two types (cleaved type and intact type), may play a role in evaluation of steatohepatitis. It has been reported that the serum level of intact CK-18 has higher predictive value than its cleaved form for the diagnosis of this disease. In the same study, intact CK-18, considering a cutoff point of 384.3, had sensitivity, specificity, PPV and NPV of 63.64, 89.36, 73.7 and 84.0, respectively; however, when the cutoff was increased to 545, its specificity and PPV increased to 95.74 and 80, respectively, and when the cutoff was decreased to 242.9, its sensitivity and NPV changed to 86.36 and 91.2, respectively[107].

Considering cleaved CK-18 as an apoptosis marker, intact CK-18 as a total apoptosis marker and cleaved CK-18-intact CK-18 as a necrosis marker[56], it seems logical that patients with steatohepatitis have higher levels of all of these 3 markers. Furthermore, it has been suggested that tissue polypeptide-specific antigen may be a serologic mirror for CK-18, so that it may also be also considered as an index for diagnosis of NASH (sensitivity, specificity, PPV and NPV of 95, 95.8, 22 and 0.09, respectively, with the cutoff value of 88 ng/mL)[135].

In sum, considering the validation of use of plasma CK-18 fragment in different studies, it can be claimed that all pieces of evidence have favored the usefulness of this index to differentiate between steatohepatitis and simple steatosis[140,141]. Therefore, some researchers believe that this biomarker can be applied as an ideal index for noninvasive diagnosis of steatohepatitis in the future, although it is not routinely accessible as a common laboratory test presently[72].

Hyaluronic acid

Hyaluronic acid is a component of extracellular matrix that is produced by mesenchymal cells[48]. Its serum level in hepatic diseases is affected by production of collagen in liver and reduction of endothelial function of hepatic sinusoids[64]. It has been shown that serum level of hyaluronic acid is significantly higher in patients with steatohepatitis, compared to healthy controls, and that it is also higher in cirrhotic patients than in NASH patients[127].

Another study conducted to investigate the possibility of using hyaluronic acid for diagnosis of steatohepatitis reported an association between this marker and stage of NAFLD[140]. Yet another study has reported that cutoff value of greater or equal to 43 has sensitivity, specificity, PPV and NPV of 65.7%, 90.5%, 92% and 61.3%, respectively, for diagnosis of NASH[142]. Moreover, hyaluronic acid has been introduced in different studies as a good and reliable index for diagnosis of hepatic fibrosis[121,143,144]. A study which was conducted to investigate accuracy of serum hyaluronic acid in prediction of hepatic fibrosis intensity in NAFLD patients showed that this marker had a positive correlation with fibrosis degree. In this study, the cutoff point of 46.1 mg/L for serum hyaluronic acid demonstrated sensitivity, specificity, PPV and NPV of 85%, 80%, 51% and 96%, respectively, for diagnosis of fibrosis, and it was concluded that measurement of serum hyaluronic acid could be a useful tool for diagnosis of patients with severe fibrosis. Besides, the logarithm of hyaluronic acid in NAFLD patients was shown to be associated with the degree of hepatic fibrosis, age and serum albumin, and it was calculated as follows: [40.1 + 0.333 × (degrees of hepatic fibrosis) + 0.032 × (age)

- 0.561 × (serum albumin)].

Based on this model, an increase of fibrosis by one degree is accompanied by an increase of serum level of hyaluronic acid by 40%[64]. In another study, hyaluronic acid was demonstrated to have NPV of 100% for the diagnosis of patients without fibrosis, when considering a cutoff value of 42 ng/mL. Sensitivity, specificity and PPV of this marker were calculated as 100%, 89% and 77%, respectively[48]. Also, another investigation in this field has suggested hyaluronic acid and type Ⅳ collagen 7s as two independent predictors of severe fibrosis, with both capable of reflecting the degree and severity of hepatic fibrosis. In this study, the mean serum level of hyaluronic acid in patients with mild fibrosis was reported as 22 ng/mL, while it was 118 ng/mL in patients with severe fibrosis, and there was statistically significance[48].

In another study by Palekar *et al*[57], hyaluronic acid of more than 45.3 mcg/L was introduced as a good index for predicting advanced hepatic fibrosis and was regarded as the strongest independent predictor of severe fibrosis among some variables such as age, sex, AST, BMI and AST/ALT ratio[57,145]. In addition, because hyaluronic acid increases in stages 3 and 4 of hepatic fibrosis, compared to markers like platelet which usually only decreases in stage 4, hyaluronic acid can be applied for prediction of fibrosis in earlier stages[72]. Owing to its importance, it has been used in different diagnostic panels[13,143,146].

In contrast, in a study investigating the clinical application of this serum marker in diagnosis of steatohepatitis, hyaluronic acid was not approved to be suitable for diagnosis of this disease[140]. In another study, this index did not show a correlation with degree of fibrosis, compared to age, TNF-α and serum concentration of type Ⅳ collagen, which had a weak but significant association; although, it could probably be used to differentiate between mild (degrees 1 and 2) and advanced fibrosis[147].

In sum, it seems that serum level of hyaluronic acid is independently and significantly related to the presence of steatohepatitis and severe fibrosis[143], and it probably can be used as a suitable marker for monitoring the progression of fibrosis toward cirrhosis in NAFLD patients[126].

Collagen 7s

Collagen 7s is another diagnostic marker that has been evaluated in different studies. Sakugawa *et al*[143] conducted research on the utility of the fibrosis marker of type Ⅵ collagen 7s for the diagnosis of steatohepatitis and reported an association between this index and the stage of fatty liver. To our knowledge, this is the only study conducted on type Ⅵ collagen 7s that has been referred to in different publications[141,148]. This marker has positive predictive value of 86% and 68.4%, negative predictive value of 61.8% and 83.6%, sensitivity of 70% and 81.3% and specificity of 81% and 71.4%, respectively, for diagnosing steatohepatitis and severe fibrosis when the cutoff point of ⩾ 5 ng/mL is considered.

Multiple studies have focused on type Ⅳ collagen 7s[76,98,112,149]. A strong and stable association between type Ⅳ collagen 7s and advanced fibrosis (before progress to cirrhosis) has been shown in different studies of patients with steatohepatitis[94]. A study by Yoneda *et al*[94] in 2007 showed that liver stiffness values had a suitable relationship with stage of hepatic fibrosis in patients with NAFLD and liver stiffness had a correlation with serum level of type Ⅳ collagen 7s. Type Ⅳ collagen 7s has been applied in different panels, such as NAFIC[98,104].

It seems that this marker is independently and significantly related to the presence of steatohepatitis and severe fibrosis and can be useful for differentiating between mild and severe forms of NAFLD[76,143].

OTHER MARKERS

Dehydroepiandrosterone

Dehydroepiandrosterone (DHEA) is one of the adrenal hormones with anti-oxidative stress effects that reduces resistance to insulin and is effective in mediating the expression of peroxisome proliferator-activated receptor alpha and mRNA of pro-collagen[149,150]. This hormone represent the most abundant steroid hormone in the body, and its interchangeable sulfated form (DHEA-S) has different functions, and influences on obesity, diabetes, atherosclerosis and osteoporosis[1,23,94].

Some studies have shown that body concentration of DHEA-S is independently and reversely correlated with death (resulting from all causes)[1]. It has also been shown that serum levels of DHEA-S have positive correlation with Hb, platelet, ALT, cholinesterase, albumin and triglyceride, and have negative correlation with age, AST, AST/ALT ratio, ALP, HDL-cholesterol, hyaluronic acid and type Ⅳ collagen 7s[65,149,150]. Thus, some researchers believe that DHEA may play a role in the pathophysiology of NAFLD and also in its progression toward more advanced stages of the disease *via* different mechanisms[23].

A study on menopausal women by Saruç *et al*[151] showed that women suffering from NAFLD had higher levels of DHEA and DHEAS than the control group (menopausal women with normal histology). In this study, DHEA and DHEAS showed positive correlation with BMI and waist circumference. A cross-sectional study conducted in 2010 on 1912 men demonstrated that steatohepatitis was associated with higher levels of serum DHEA-S, and the highest risk of NASH development was reported for those who had the highest serum level of DHEA-S (OR = 1.59, 95%CI: 1.04-2.43)[152]. Subsequently, a study conducted to investigate the clinical significance of serum level of DHEA-S in NAFLD patients showed that the serum level of this hormone was significantly higher in patients than in controls[23].

The study by Sumida *et al*[65] reported the same results, although the difference did not reach statistical significance (128.7 μg/dL in NAFLD patients *vs* 113.6 μg/dL in control group). However, this study demonstrated that patients suffering from severe stages of NAFLD (steatohepatitis with fibrosis of stage 3 or 4) had lower serum level of DHEA-S than patients with mild stages of the disease (simple steatosis or steatohepatitis with fibrosis of stage 0-2)[65].

In parallel, some other studies have also shown that NASH with advanced fibrosis is strongly associated with low concentration of circulating DHEA-S[149,153]. This association persisted even after adjustment for variables such as age, sex and resistance to insulin[65]. By applying single-variable analysis of serum level of DHEA in another study, a significant difference was detected between mild fibrosis and advanced stages of steatohepatitis (95 ± 9 μg/dL *vs* 72 ± 28 μg/dL); however, this marker was unable to differentiate between patients and non-patients[153]. These results confirmed the results of another study conducted 4 years earlier which had shown that the mean DHEA-S level in patients with degree 2-4 of steatohepatitis was significantly lower than in those suffering from milder stages (0-1) and that the mean of DHEA-S level decreased stage by stage with increase in the fibrosis stages. In this study, a DHEA-S value of more than 1.0 μg/mL showed sensitivity of 95% and specificity of 58% for diagnosis of advanced stages of NAFLD[149]. In another study, sensitivity, specificity, PPV and NPV were 76.5%, 73.3%, 29.5% and 95.5%, respectively, considering a cutoff point of equal or less than 66 mg/dL[65]. In the same direction, Sumida *et al*[1] in another article suggested that patients with DHEA-S serum level of more than 66 μg/dL are unlikely to suffer from advanced stages of NAFLD.

It can be generally concluded that increased level of serum DHEA-S may be a part of the pathophysiology of NAFLD and could play a role in the development of this disease[23]. However, considering metabolic and intracellular effects reported for this hormone, such as its protective effect against oxidative damage in animal models which is applied by reducing concentration of MDA and increasing activity of superoxide dismutase in hepatocytes and total concentration of glutathione, it may have an important role in prevention of histological progress of NAFLD; its blood level variations may also be well correlated with different histological patterns of patients with similar metabolic profile, age and sex[149]. Considering these findings, it is not surprising if serum level of DHEA developed into a successful predictor for diagnosis and staging of hepatic fibrosis in NAFLD patients in the future.

Fibrinogen-like protein 2

Fibrinogen-like protein 2 (FGL2) is a new member of the fibrinogen-like protein family. A study investigating this protein in patients with NAFLD showed that plasma level of this protein was considerably higher in patients with steatohepatitis, but there was no difference between patients with simple steatosis and the control group. Results of this study indicated a potential role for FGL2 level in the diagnosis of severe forms of fatty liver diseases and differentiated between simple steatosis and steatohepatitis[154].

CONCLUSION

Although some of the reported markers such as adiponectin, hyaluronic acid, CK-18 and DHEA have shown promising results, at present, it does not seem that any of these individual markers are reliable enough to be considered as a marker for diagnosis and staging of NAFLD. An ideal biomarker should have specific characteristics such as simplicity, accessibility, accuracy, repeatability and cost-effectiveness, some of which are not applicable to any of these markers[72,155]. Also, a reliable marker should be able to provide physicians with clear information, have high diagnostic power for differentiation between different stages of the disease, and should have been validated in large prospective trials[156,157]. It should be noted, as well, that selection of a suitable test depends on the clinical demand of the healthcare provider.

If ruling out of the diagnosis of NAFLD and/or its staging is the goal, applying the markers with higher sensitivity and NPV is recommended; however, if the main aim is diagnosis of the disease in suspected patients, the test's specificity and PPV would be the most important factors that should be taken into consideration. Furthermore, determining a suitable cutoff point for a test is usually based on a tradeoff between sensitivity (true positive) and specificity (true negative) results of the test. In fact, the optimal cutoff point has the highest sensitivity and specificity, but this point can hardly be obtained for a marker. Therefore, based on different cutoff values, the diagnostic power of a test and its productivity will be different. Paying attention to different cutoff values suggested for each marker while comparing them is important.

Considering the weaknesses of every individual marker for diagnosis and staging of the disease, it seems that the present trend would be toward combining some of these serum markers together, and even adding some other parameters, such as demographic variables or radiologic results, and developing a mathematical model which has higher diagnostic power and accuracy[72,158].

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