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TOPIC HIGHLIGHT

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Non-alcoholic fatty liver disease and diabetes: From physiopathological interplay to diagnosis and treatment

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is highly prevalent in patients with diabetes mellitus and increasing evidence suggests that patients with type 2 diabetes are at a particularly high risk for developing the progressive forms of NAFLD, non-alcoholic steatohepatitis and associated advanced liver fibrosis. Moreover, diabetes is an independent risk factor for NAFLD progression, and for hepatocellular carcinoma development and liver-related mortality in prospective studies. Notwithstanding, patients with NAFLD have an elevated prevalence of prediabetes. Recent studies have shown that NAFLD presence predicts the development of type 2 diabetes. Diabetes and NAFLD have mutual pathogenetic mechanisms and it is possible that genetic and environmental factors interact with metabolic derangements to accelerate NAFLD progression in diabetic patients. The diagnosis of the more advanced stages of NAFLD in diabetic patients shares the same challenges as in non-diabetic patients and it includes imaging and serological methods, although histopathological evaluation is still considered the gold standard diagnostic method. An effective established treatment is not yet available for patients with steatohepatitis and fibrosis and randomized clinical trials including only diabetic patients are lacking. We sought to outline the published data including epidemiology, pathogenesis, diagnosis and treatment of NAFLD in diabetic patients, in order to better understand the interplay between these two prevalent diseases and identify the gaps that still need to be fulfilled in the management of NAFLD in patients with diabetes mellitus.

Key words: Non-alcoholic fatty liver disease; Diabetes mellitus; Pathogenesis; Diagnosis; Treatment

Core tip: This review addresses the important interplay between non-alcoholic fatty liver disease and diabetes mellitus, with particular emphasis on physiopathological mechanisms, diagnosis and treatment.

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INTRODUCTION

Diabetes mellitus (DM) is a public-health concern: 347 million people worldwide have diabetes and the WHO projects that diabetes will be the 7th leading cause of death in 2030. It is a group of metabolic diseases charac-

terized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. There are two most important etiopathogenetic categories: type 1 DM (T1DM), which accounts for 5%-10% of the cases of DM and results from autoimmune destruction of the beta-cells of the pancreas and absolute deficiency of insulin; and type 2 DM (T2DM), which encompasses patients who have insulin resistance with relative insulin deficiency and accounts for 90%-95% of diabetic patients. Both T1DM and T2DM patients are at high risk of developing chronic macrovascular and microvascular degenerative complications. Nevertheless, beyond these classic complications, DM is also associated with liver-related mortality and increasing risk of hepatocellular carcinoma[1,2].

Non-alcoholic fatty liver disease (NAFLD) prevalence is increased in patients with DM[3,4]. Increasing evidence suggests that patients with T2DM are at a particularly high risk for non-alcoholic steatohepatitis (NASH), with varying degrees of liver fibrosis[5,6]. Likewise, preexisting diabetes is an independent risk factor for NAFLD progression and for liver-related mortality and hepatocellular carcinoma in prospective studies[1,7-9]. On the other hand, patients with NAFLD have an elevated prevalence of prediabetes[10] and recent data has shown that NAFLD presence predicts the development of T2DM[10,11].

There are pathogenetic mechanisms linking NAFLD and DM. Besides insulin resistance and hyperinsulinemia, disordered lipid metabolism, increased oxidative stress and inflammation contribute to both entities. It is possible that genetic and environmental factors interact with metabolic derangements to accelerate NAFLD progression in diabetic patients.

Ultrasound is the method of choice for screening patients for NAFLD. In patients with diabetes and histologically-proven NASH, abnormal liver enzymes may be seen in less than 20% of patients[5,6,12,13]. Liver biopsy is still the gold standard method to diagnose and stage NAFLD. However, it is a costly and invasive procedure with some limitations, such as sampling error and intra-and inter-observer variability among pathologists[6]. It was previously suggested that liver biopsy should be considered in all patients with DM and hepatic steatosis on ultrasound[6]. Newer radiological techniques, biomarkers and clinical algorithms are being currently studied and may provide, in the near future, valuable noninvasive alternatives to histological diagnosis. Treatment is based mainly on lifestyle changes and antioxidants and numerous drugs have been studied searching for histological improvement with variable outcomes. The following review aims to discuss all these aspects from physiopathological mechanisms to diagnosis and treatment of NAFLD in DM.

DEFINITION OF NAFLD

In 1980, Ludwig *et al*[14] described a small series of patients with liver histology characterized by fat accumulation, hepatic necroinflammation and, in most cases, fibrosis in the absence of a history of excessive alcohol consumption. Since this original description, the histologic criterion for diagnosing NAFLD has evolved, and several grading systems have been proposed to assess histologic severity. In 1999, Matteoni *et al*[15] proposed pathological NAFLD subtypes based on long-term outcome studies, and Brunt *et al*[16] proposed a specific classification for NASH based on criteria used in other forms of chronic liver disease, using grades of necroinflammatory lesions and stages of fibrosis. Subsequently, the US Pathology Committee of the NASH Clinical Research Network[17] proposed and validated a histological scoring system of specific lesions that addressed the full spectrum of NAFLD. In addition, a separate scoring system known as the NAFLD activity score (NAS) has been developed for use in clinical trials. It measures steatosis semi-quantitatively, as well as lobular inflammation and hepatocellular ballooning, to enable systematic documentation of changes in activity of NASH[17]. However, a numerical value alone is not accepted for definitive diagnosis of steatohepatitis. In general, accumulation of greater than 5% of fat, particularly in the form of triglycerides, is considered to be the minimal requirement for histologic diagnosis of steatosis. NASH, in turn, encompasses steatosis plus lobular inflammation and ballooning degeneration, with or without a varying degrees of fibrosis, which is originally centered on the hepatic venulae.

CLINICAL PRESENTATION

NAFLD is generally asymptomatic at presentation and is frequently found among individuals with conditions such as obesity, T2DM, metabolic syndrome and its individual components[18]. The most common signs and symptoms are fatigue, right upper quadrant pain and hepatomegaly, as well as acanthosis nigricans, which are more frequently observed in the pediatric population. In fact, most patients with NAFLD are diagnosed by incidental elevated liver enzymes or imaging studies suggesting hepatic steatosis[18]. By definition, NAFLD is established in patients who consume little or no alcohol presenting a histological picture that resembles alcohol-induced liver disease. Most studies defined a threshold for excessive alcohol consumption as more than 20 g/d in women and more than 30 g/d in men[15,19]. In contrast, intake levels of 20 g/d (140 g weekly) for men, and 10 g/d (70 g weekly) for women have been endorsed as the acceptable thresholds to define "non-alcoholic" in the guidelines proposed by the Asia-Pacific Working Party for NAFLD (APWP-NAFLD) and by the National Institutes of Health Clinical Research Network[20,21]. Additionally, definitive diagnosis of NAFLD requires exclusion of other secondary causes of hepatic steatosis, such as medications like prednisolone, tamoxifen, amiodarone and methotrexate among others, exposure to toxins as vinyl chloride, total parenteral nutrition, cachexia, intestinal bypass surgery, viruses infections like genotype 3 hepatitis C virus and human immunodeficiency virus.

Table 1 Studies with histopathological evaluation of diabetic patients with non-alcoholic fatty liver disease							
Ref.	Sample size	Metabolic syndrome and its components	Diabetes duration (yr)	Diabetes-related complications	Elevated enzyme levels AST/ALT/GGT	Risk factors for NASH	Fibrosis
Amarapurka et al[12]	36						30.5%
Gupte et al[13]	32				ALT and/or AST 31%	No risk factors	22%
Kemmer et al[38]	22 (females)					No risk factors	
Leite et al[6]	92	Hypertension 88%	7.8	Microvascular 46%	14%/16%/13%	Hypertriglyceridemia High ALT	34%-60%
		Dyslipidaemia 86%		Macrovaascular 26%		Low HDL-chol	
Prashanth et al[5]	83	Metabolic syndrome 77%	8.2		ALT 7%	MS components High ALT High AP	37%

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gammaglutamyltransferase; AP: Alkaline phosphatase; NASH: Non-alcoholic steatohepatitis; HDL-chol: HDL-cholesterol.

EPIDEMIOLOGY

NAFLD is the commonest cause of liver disease in western countries, present in over 30% of the general population[22,23]. The prevalence in Asian populations ranges from 6% up to 25%[24]. The prevalence of NAFLD among children is 3%-10%, rising up to 40%-70% among obese children[25]. The prevalence of NASH is difficult to assess because it requires a histological diagnosis, which is impractical in all patients with NAFLD. In a recent study in a cohort of 400 military personnel and their relatives, ultrasound prevalence of NAFLD was 46%, and NASH was found in 30% of these individuals, resulting in a NASH prevalence of 12% for the entire cohort[26].

NASH cirrhosis is now the third most common indication for liver transplantation in the United States, behind hepatitis C and alcoholic liver disease, and is the only liver-related transplant indication that continues to increase[27]. Liver disease is the third leading cause of death in NAFLD patients[8,28], with hepatocellular carcinoma being the most frequent cause of liver-related death[29].

T2DM is not only a risk factor for NAFLD but it is also related to a higher prevalence of NASH and fibrosis[1,5,6]. Data regarding T1DM and NAFLD are scarce. Although T1DM is characterized by insulin deficiency, obesity and metabolic syndrome may eventually occur in these patients and lead to NAFLD[30]. Targher et al[31] found a NAFLD prevalence of 44% in patients with T1DM by ultrasound imaging, without histological confirmation. Nevertheless, the prevalence of NAFLD in patients with T2DM has been reported to be as high as 74%[3,4,26,32,33]. Most of the studies evaluating NAFLD in T2DM have relied exclusively on ultrasonography or on elevated liver enzymes for diagnosis. Studies describing the histopathological spectrum of NAFLD in type 2 diabetes mellitus are still scarce[6].

NAFLD AND DIABETES MELLITUS: THE NATURAL HISTORY IS A TWO-WAY TRAFFIC

NAFLD represents a wide spectrum of conditions ranging from fatty liver, which follows in general a benign and stable clinical course, to NASH that may progress to cirrhosis. Less than 1% to 4% of patients with simple steatosis progress to advanced fibrosis. By contrast, NASH can lead to cirrhosis in 15% to 25% of individuals, with further liver-related complications and death[8,11,34]. Among those with NASH-related cirrhosis, about 25% will develop major complications of portal hypertension within 3 years[35].

The role of diabetes in NASH and fibrosis was initially evaluated in patients undergoing bariatric surgery. The prevalence of T2DM ranged from 14% to 28% in these studies and its presence was a predictor of NASH and fibrosis in morbidly obese patients[36,37].

Younossi et al[1], in a retrospective cohort study with 132 patients with histological diagnosis of NAFLD, reported that patients with DM had greater rates of cirrhosis and mortality than those with NAFLD without DM. In a recent prospective study of 328 asymptomatic patients, 16.5% had an established diagnosis of T2DM. The prevalence of NAFLD and NASH in the entire cohort was 46% and 12%, respectively. However, in diabetic patients, NAFLD was observed in 74% and NASH in 22%[26].

Few studies evaluated the prevalence of NAFLD and the correlated factors with histopathological stages of NAFLD in patients with T2DM. Table 1 outlines a summary of these studies. The sample sizes varied from 32 to 92 patients, and NASH was present in 63%-87% of the patients, while the prevalence of any fibrosis ranged from 22% to 60% in these studies[5,6,13]. In the largest single-centre study, we found high prevalence of the more severe stages of NAFLD: 78% for NASH and 34%-60% for moderate-to-severe fibrosis[6]. No diabetes-related variable, such as glycemic control, diabetes duration or the presence of micro- and macro-vascular complications, was associated with the more severe stages of NAFLD[6]. Alanine aminotransferase (ALT), high triglycerides and low HDL-cholesterol were independently associated with NASH. On the other hand, male gender, older age and elevated values of gammaglutamyl transferase (GGT) were associated with moderate-to-

severe fibrosis[6]. Prashanth *et al*[5] reported prevalence of NASH and of any stage of fibrosis of 62.6% and 37.3%, respectively. Serum ALT and alkaline phosphatase levels, although within normal limits, were significantly higher in patients with NASH[5]. Of note, NASH prevalence increased proportionally to the number of metabolic syndrome components presented. Also, no diabetes-related variable was predictive of the severity of NAFLD. In a study conducted in Mexico, 60 patients with DM were evaluated and 22 of them (37%) had elevated liver enzymes and/or steatosis on radiological examination[38]. These patients underwent liver biopsy and the prevalence of NASH was 64%. There was no association of liver enzymes, lipid profile, glycated hemoglobin or body mass index with the presence of NASH. In another small group of 32 T2DM patients submitted to liver biopsies, 49% had NAFLD on histopathology, of which 87.5% had NASH. There was no significant correlation between liver enzymes and NASH or fibrosis[13]. Subsequently, these researchers further reported that in 36 patients with NASH, 30.5% had any stage of fibrosis. Patients with any stage of fibrosis had higher levels of aminotransferases and a higher aspartate aminotransferase (AST) to ALT ratio[12].

Although in most of the studies diabetic patients with NASH had higher serum ALT than those without NASH, and ALT levels were independently associated with the presence of NASH on liver biopsy, abnormal ALT levels were uncommon and did not have enough predictive value to be indicated as a screening test for NASH detection[6]. Moreover, in patients with T2DM, serum liver enzymes could be less representative of the severity of intrahepatic fat accumulation. In a case-control study, patients with T2DM showed approximately 80% more intra-hepatic fat content by magnetic resonance spectroscopy than age, sex, and body weight-matched non-diabetic controls[39]. Liver fat content was underestimated by serum ALT compared with equally obese non-diabetic subjects.

A recent cross-sectional study demonstrated that in addition to diabetes, a family history of diabetes also increased the risk of NASH and fibrosis in non-diabetic individuals[40]. The association between family history of diabetes with NASH and fibrosis remained significant even after adjusting for prediabetes status, suggesting that a family history of diabetes may provide additional risk stratification in non-diabetic patients with NAFLD.

In spite of apparent absence of association between any diabetes-related characteristic and the presence of more severe stages of NAFLD in previous studies, evidence is mounting that NAFLD may be associated with the occurrence of microvascular and macrovascular degenerative complications in diabetic patients[3,31,41,42]. In patients with T1DM and T2DM, NAFLD was associated with higher rates of microalbuminuria, reduced glomerular filtration rate and retinopathy[43,44]. Prospective studies have demonstrated a higher incidence of chronic kidney disease in patients with T2DM and NAFLD, independent

of several established risk factors[42,43]. Both subclinical atherosclerosis markers, like increased carotid intima-media thickness and aortic stiffness, as well as clinical cardiovascular diseases, were also more frequent in patients with T1DM and T2DM with the diagnosis of NAFLD than in their counterparts without NAFLD[3,31,45]. It was demonstrated that the association between macrovascular disease and NAFLD was independent of classic cardiovascular risk factors and components of metabolic syndrome[3,31].

Another issue to be considered is whether diabetes also accelerates the progression of NAFLD. The presence of DM was independently associated with advanced liver fibrosis in cross-sectional studies[46,47]. However, findings from prospective studies with serial liver biopsies are still scarce and controversial. In a cohort study with 129 patients with biopsy-proven NAFLD reevaluated after a mean follow-up of 13.7 years, the progression of liver fibrosis occurred in 41%[8]. More pronounced insulin resistance during follow-up was associated with liver fibrosis progression. Unfortunately, fasting plasma glucose was not measured at baseline, and thus the prevalence of diabetes at onset of the study could not be reported[8]. In another cohort of 103 NAFLD patients who underwent a second liver biopsy at an average interval of 3.2 years, fibrosis staging progressed in 37%. Preexisting diabetes and early stages of fibrosis at first biopsy were predictors of fibrosis progression[7]. Otherwise, in a systematic review including ten longitudinal studies comprising 221 patients who had NASH on their initial biopsy, received no intervention of proven benefit regarding histology and underwent a second liver biopsy at least one year apart, only age and any degree of inflammation in the initial biopsy were the risk factors related to progression to advanced fibrosis. Other traditional parameters such as obesity, diabetes and hypertension were not statistically significant predictors[48]. Overall, inclusion of heterogeneous studies, disagreement on criteria for NASH diagnosis, liver biopsy sampling error and variability among pathologists are remarkable limitations of long-term histological studies.

It is not clearly defined yet if NAFLD worsens glycemic control in patients with T2DM. NAFLD patients have shown both an impaired ability of insulin to suppress endogenous glucose production related to hepatic insulin resistance, and a reduction in glucose disposal, a measure of whole-body insulin sensitivity[49]. Indeed, intrahepatic triglyceride content may influence insulin requirements in diabetic patients *via* an effect on the sensitivity of endogenous glucose production to insulin[50].

On the other hand, NAFLD is known to be associated with insulin resistance, hyperinsulinemia and prediabetes, which includes both impaired fasting glucose (IFG) and impaired glucose tolerance (IGT). In an Australian study of 70 patients with NAFLD determined by ultrasound, 24% had IGT and 10% had diabetes on standard oral glucose tolerance test (OGTT). Over half of the NAFLD patients with a normal fasting glucose had
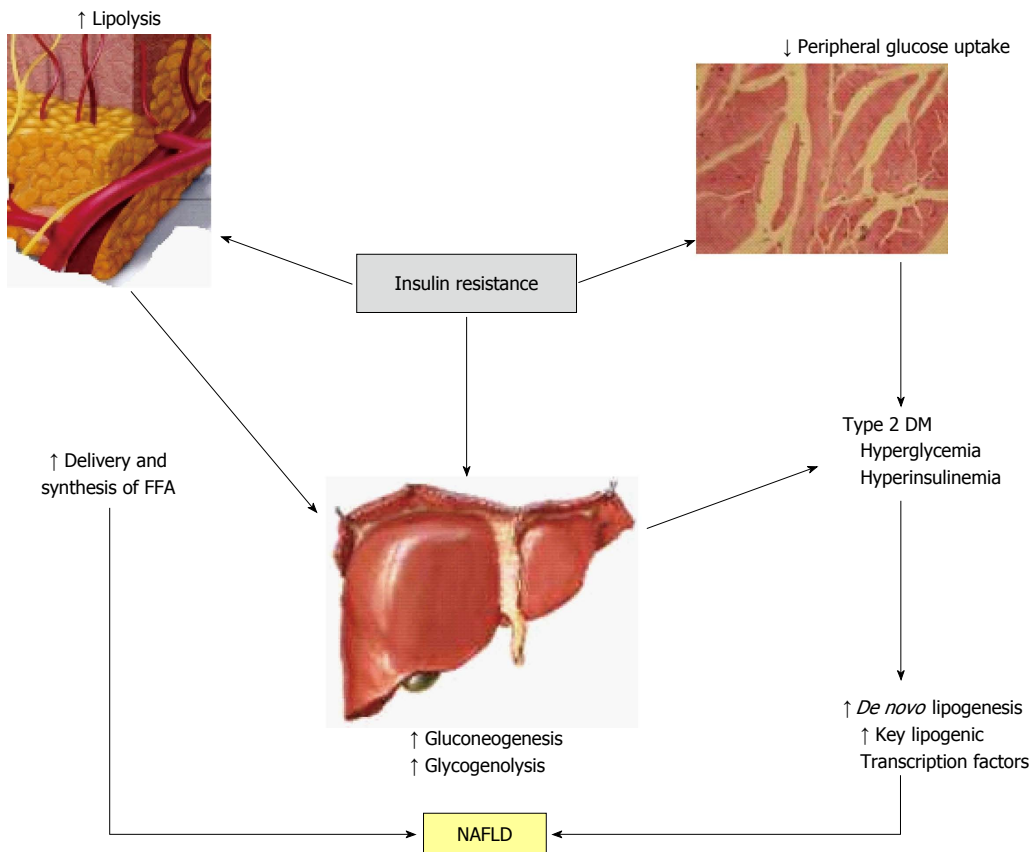
Figure 1 Pivotal role of insulin resistance in non-alcoholic fatty liver disease pathophysiology. ↓: Decrease; ↑: Increase. FFA: Free fatty acids; DM: Diabetes mellitus; NAFLD: Non-alcoholic fatty liver disease.

abnormal glucose tolerance, as detected by OGTT. In addition, irrespective of the status of glucose tolerance, 2-h hyperinsulinemia during the OGTT occurred in all subjects with NAFLD, and fasting insulin resistance was found in 73%; fasting insulin resistance was assessed by the homeostasis model assessment method (HOMA-IR) and was defined by a HOMA-IR equal or greater than 2[51]. In the Framingham Heart study with 2589 individuals, fatty liver was associated with T2DM, IFG, hypertension, metabolic syndrome, HDL-cholesterol, triglycerides, and adiponectin levels, even after multivariate adjustment for other fat depots, such as visceral adipose tissue, waist circumference, and body mass index[52]. Moreover, several prospective observational studies have shown an increased incidence of T2DM in patients with NAFLD diagnosed by ultrasonography or by liver biopsy. However, most of them were not adjusted for main covariates, such as family history of T2DM, physical activity and fasting glucose and insulin levels[8,53].

In a recent prospective community-based study in an urban adult population from Sri Lanka, individuals with ultrasonographic NAFLD showed an increased risk of developing T2DM. After three years follow-up, T2DM incidence rates were 64.2 and 34 per 1000 person/years for those with and without NAFLD, respectively, and NAFLD was an independent predictor of T2DM development[54]. In a longitudinal cohort study of 7849 Korean individuals followed-up for 5 years, those who had

both elevated ALT and ultrasonographic liver steatosis had an increased risk of future diabetes development[55]. Another Korean study demonstrated that NAFLD had an independent and additive effect on the development of T2DM in patients with IFG at baseline[56]. Similarly, Musso *et al*[11] reported an increased risk for incident diabetes in patients with evidence of ultrasonographic and histological NAFLD in a recent meta-analysis of three large community-based cohorts with a range of follow-up from 4 to 10 years.

Overall, it appears that DM and NAFLD are two conditions with intense interplay roles, one adversely affecting the natural history of the other, and vice-versa.

PATHOGENETIC MECHANISMS

The pathophysiological hallmark of NAFLD is the underlying insulin resistance (Figure 1). Insulin resistance at the level of the adipocyte seems to be the primary defect in NAFLD, leading to increased lipolysis. Any grade of insulin secretion deficit associated with DM further increases lipase activity in adipose tissue. This leads first to elevated circulating and portal free fatty acids (FFAs) and subsequently to their increased skeletal muscle and hepatic delivery and uptake, which decreases insulin action in these tissues[57,58]. Insulin resistance in these tissues leads to increased gluconeogenesis and glycogenolysis in liver as well as reduction in peripheral glucose disposal result-

ing in hyperglycemia. The pancreatic beta islet cells adapt to hyperglycemia by increasing insulin secretion, leading to hyperinsulinemia. Hyperinsulinemia and hyperglycemia also upregulate several key lipogenic transcription factors, including sterol regulatory element-binding protein 1c (SREBP1c) and carbohydrate response element binding protein (ChREBP), promoting hepatic lipid synthesis or *de novo* lipogenesis[59]. In patients with NAFLD, studies have shown that the vast majority of hepatic fat originates from FFAs (59%), but 26% comes from *de novo* lipogenesis and 15% originates from the diet[60].

Hepatic steatosis results when the balance between delivery and synthesis of FFAs exceeds the liver capacity to oxidize or export them. Lipids are exported in the form of very-low-density lipoprotein (VLDL), and the synthesis of apolipoprotein B-100, which is the apolipoprotein contained in VLDL particles, is reduced in patients with NASH[61]. Accumulation of lipids can exert toxic effects on the liver by inefficient oxidation or activation of inflammatory pathways. Although hepatic triglycerides (triacylglycerol) are thought to be protective for NAFLD progression, certain lipid metabolites such as diacylglycerol and ceramides may themselves cause cell injury and death and contribute to NASH development[62].

FFAs may be oxidized within mitochondria, peroxisomes and microsomal system. Increased FFA oxidation causes an oxidative stress that further uncouples mitochondrial oxidation/phosphorylation and generates more reactive oxygen species (ROS). Indeed, abnormal mitochondrial morphology and function is frequently present in the hepatocytes of patients with NASH. These abnormalities might render hepatocytes even more susceptible to oxidative damage[63]. Oxidative stress generated from ROS promotes lipid peroxidation and augments inflammation by up-regulating key factors and pathways of NF-κB and toll-like receptor (TLR) signaling[64].

Systemic subclinical inflammation also appears to be involved in the pathogenesis of NAFLD and NASH. Patients with NAFLD have higher circulating markers of inflammation than healthy controls. It has been shown that diabetic patients with NAFLD have higher circulating markers of inflammation than diabetic patients without NAFLD[65]. It has been also described that, as obesity increases, there is an increase in macrophage infiltration in adipose tissue. These activated macrophages secrete inflammatory cytokines such as TNF-alpha and IL-6 that may exacerbate insulin resistance by decreasing insulin signaling. In turn, serum levels of adiponectin, an anti-inflammatory insulin sensitizing and potentially hepatoprotective adipokine, are reduced in patients with NAFLD and are lower in NASH than in simple steatosis[66,67]. Furthermore, adiponectin levels fall in prediabetes and in T2DM and there could be a link between adipocyte dysfunction and NASH in these patients[68]. It is now recognized that NAFLD and particularly NASH progression results from a complex interplay between insulin resistance with hyperinsulinemia, increased oxidative stress, hepatic and systemic inflammation. Hyper-

insulinemia combined with ongoing liver inflammation and hepatocyte apoptosis may induce profibrotic factors. Thus, profibrotic factors can contribute to fibrosis progression by activating hepatocyte stellate cells.

NAFLD has recently been linked to alterations of gut microbiota and its metabolic effects. Increased absorption of lipopolysaccharides (LPS) resulting from a "leaky" small intestinal mucosa may cause activation of the innate immune system, by direct stimulation of TLR-signaling, leading to inflammation and insulin resistance[69]. Of note, patients with T2DM had mean plasma levels of LPS higher than controls due to a higher small intestinal bacterial overgrowth and increased leakiness of the intestinal mucosa[70].

Genetic factors may also play a role in the development of NAFLD. Based on the complex mechanisms involved in the pathogenesis of NAFLD, there is a low likelihood of finding a single candidate gene responsible for NAFLD or a clear genetic link between T2DM and NAFLD. Through several genome-wide association studies, the missense rs738409 C/G single-nucleotide polymorphism implying an amino acid change from isoleucine (I) to methionine (M) at the position 148 (I148M) of the protein encoding by the *patatin-like phospholipase domain-containing 3* gene (*PNPLA3*) was strongly associated with increased hepatic fat content and NAFLD histological severity[71,72]. Genetic variation at *PNPLA3* seems to confer a markedly increased risk of severe histological features of NAFLD, but there is no association of this genetic polymorphism with body mass index, triglyceride, HDL- and LDL-cholesterol levels, or diabetes[73]. Other polymorphisms in the microsomal triglyceride transfer protein or in the gene of superoxide dismutase 2 could play a role in the interaction between NAFLD and diabetes[74,75].

The main physiopathological mechanisms involved in NAFLD progression from simple steatosis to NASH and fibrosis are summarized in Figure 2.

DIAGNOSIS

When NAFLD is suspected, the first step to define its diagnosis is to exclude other known etiologies of chronic liver diseases like drug-related steatosis[76,77], viruses and alcohol. As previously described, a careful history of alcohol ingestion must be taken. Of note, diabetic patients with excessive alcohol intake may have both alcoholic- and NAFLD[78].

Liver enzymes may be elevated, but normal aminotransferases do not exclude the diagnosis of NAFLD, even in diabetic individuals[4,79]. Aminotransferase levels have been reevaluated and new thresholds have been suggested for normal levels when considering patients with NAFLD. These levels are 19 U/L for men and 30 U/L for women and this has improved the sensitivity for diagnosing NAFLD[80], although the diagnosis of NASH still cannot be performed based solely on aminotransferases. Although unspecific, serum ferritin levels may be high and it is important to discard hemochromatosis
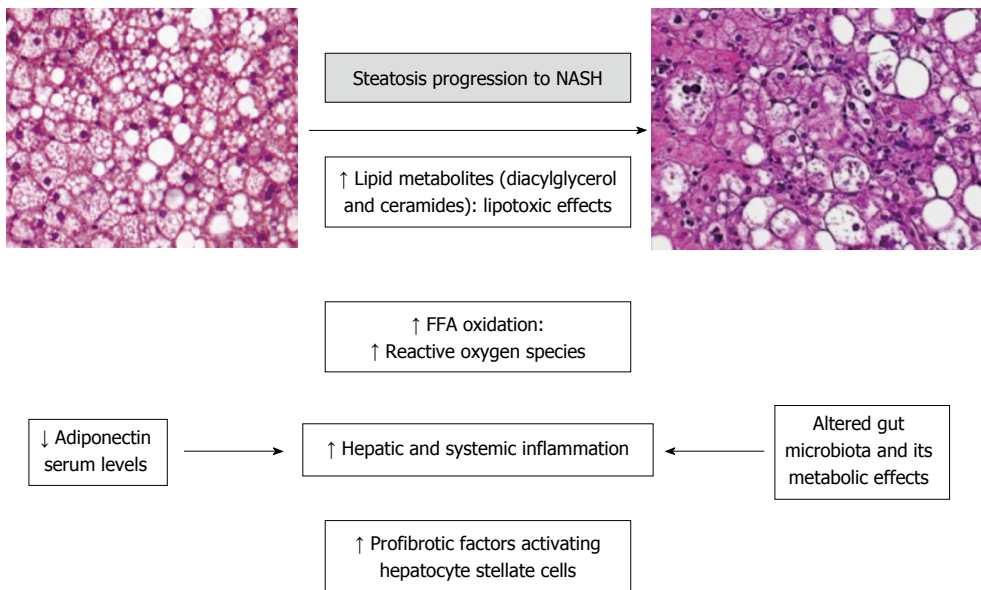
Figure 2 Main physiopathological mechanisms influencing non-alcoholic fatty liver disease. Progression to non-alcoholic steatohepatitis and fibrosis. ↓: Decrease; ↑: Increase. NASH: Non-alcoholic steatohepatitis; FFA: Free fatty acids.

in patients with a high transferrin saturation index[81]. Low-titer autoantibodies, such as anti-nuclear and anti-smooth muscle, can be found as an epiphenomenon in NAFLD[82], although a liver biopsy may be indicated to exclude autoimmune liver disease.

The diagnostic approach to patients with NAFLD is based mainly on imaging, serological and histopathological methods. Apart from DM, other clinical conditions are associated with NAFLD, such as essential hypertension, obesity, hypertriglyceridemia, polycystic ovary disease and metabolic syndrome[83-85]. Thus, NAFLD should also be investigated in these clinical settings, and routinely in DM.

The spectrum of NAFLD is similar in diabetic and non-diabetic individuals, and it develops from simple steatosis to advanced fibrosis, cirrhosis and hepatocellular carcinoma[86]. The only reliable method that identifies these different stages is liver biopsy. However, owing to its potential complications and limitations like cost, sampling error and procedure risks, many non-invasive methods have been proposed to diagnose NAFLD and to predict those patients with a higher risk of having NASH[87,88].

Imaging methods have a variable accuracy to identify liver steatosis[89]. Liver ultrasonography (US) is a safe, inexpensive and readily available method. It is the most used technique to diagnose NAFLD with a sensitivity of 60%-94% and a specificity of 66%-95% for detecting steatosis[90,91]. Its main limitation is that it is operator-dependent and cannot detect mild steatosis (5%-30%)[90-92]. Additional Doppler fluxometry helps identify indirect signs of advanced liver disease. Recently, Ballestri *et al*[93] developed the ultrasonographic fatty liver indicator (US-FLI), a new score ranging from 2 to 8 points, which is capable of ruling out NASH based on US parameters like the intensity of liver and kidney contrast, posterior attenuation of US beam, vessel blurring, difficult visual-ization of gallbladder wall, difficult visualization of the diaphragm and areas of focal sparing. An US-FLI < 4 has a negative predictive value for NASH of 94% and can be easily assessed[93].

Computed tomography (CT) allows quantitative and qualitative evaluation of liver steatosis with a higher accuracy. Based on the difference of the hepatic-splenic attenuation, unenhanced CT can detect liver steatosis grades as low as 5%[94]. Magnetic resonance (MR) imaging with appropriate sequences also provides high sensitivity and specificity. MR spectroscopy is one of the most accurate methods for the evaluation of liver steatosis, has a strong correlation with histology and can detect very low levels of steatosis[95]. Recently, MR elastography has showed a high predictive value for excluding advanced fibrosis and a good accuracy for detecting NASH with an area under ROC curve of 0.93. MR elastography discriminated NASH from steatosis with a sensitivity of 94% and specificity of 73% with a cutoff of 2.74 kilopascals units (kPa)[96]. However, MR is too expensive to be used routinely, but might be useful in patients under study protocols and in those with a strong suspicious of NAFLD with normal liver echogenicity on ultrasound[97].

A novel method to diagnose and quantify steatosis is the controlled attenuated parameter (CAP)[98]. CAP is software that can be used simultaneously with liver transient elastography available by Fibroscan[99,100]. It is a simple and easily performed method that can detect liver steatosis at levels as low as 5%. Sasso *et al*[99] defined the best cutoff value of 292 for severe steatosis (> 66%) detection, with a negative predictive value of 100%. Its main limitation is the difficulty of obtaining reliable measurements in obese patients. When liver steatosis is estimated using CAP, liver stiffness is also evaluated by Fibroscan elastography. In this method, vibrations of mild amplitude and low frequency are transmitted by the

transducer, inducing an elastic shear wave that propagates through the liver. The velocity of wave propagation relates directly to liver stiffness or fibrosis: the stiffer the tissue, the faster the shear wave propagates. The velocity of the shear wave propagation is measured in kilopascals (kPa). Higher tissue stiffness corresponds to increasing severity of fibrosis. Wong *et al*[101] defined a cutoff of 10.3 kPa in NAFLD patients to predict advanced fibrosis with a sensitivity of 92% and specificity of 88%. The negative predictive value of this cutoff for advanced fibrosis was 99%. The possibility of evaluating fibrosis and steatosis simultaneously makes the Fibroscan a valuable tool in the study of NAFLD.

Many serological methods have been evaluated in the diagnosis of NAFLD regarding their accuracy for detecting NASH[102]. In this setting, the AST/ALT ratio[103], FIB-4 index[87], the BARD score[47], NAFLD Fibrosis Score[46], and the enhanced liver fibrosis (ELF) test shall be addressed. The AST/ALT ratio has been used to identify patients with advanced fibrosis and a value > 1 may predict advanced fibrosis in patients with NAFLD[103]. The FIB-4 index is easily calculated using the following formula: [age (years) × AST (U/L)]/[platelet (10^9/L) × square root of ALT (U/L)] being useful for the diagnosis of liver fibrosis, but not capable of diagnosing NASH. It was described that a FIB-4 index $\geq$ 2.67 had an 80% positive predictive value and a FIB-4 index $\leq$ 1.30 had a 90% negative predictive value for fibrosis in patients with NAFLD, although other non-invasive tests like the ELF and Fibrotest were more accurate than FIB-4 index to predict advanced fibrosis[87,104]. The NAFLD fibrosis score and the BARD score include DM as a variable in their formula. The BARD score is composed of three variables: an AST/ALT ratio $\geq$ 0.8 sums 2 points; a BMI $\geq$ 28 sums 1 point; presence of diabetes sums 1 point. The possible score ranges from 0 to 4 points. According to the results by Harrison *et al*[47], score values of 0 or 1 would have a high negative predictive value (NPV) for severe fibrosis. The NAFLD score comprises demographic and easily obtained laboratory variables as age, BMI, hyperglycemia, platelet count, albumin and AST/ALT ratio. The formulae {-1.675 + [0.037 × age (years)] + (0.094 × BMI) + [1.13 × IFG/diabetes (yes = 1, no = 0)] + (0.99 × AST/ALT) - [0.013 × platelet (10^9/L)] - [0.66 × albumin (g/dL)]} can be assessed online at http://NAFLDscore. com. According to several studies it has a high accuracy to predict advanced fibrosis. Musso *et al*[11] showed in a meta-analysis that the NAFLD score had a sensitivity and specificity higher than 90% to predict advanced fibrosis and suggested that the combination of two non-invasive methods like the NAFLD score and Fibroscan elastography could be a useful tool in this setting. The ELF panel, obtained through the assessment of three matrix turnover proteins (hyaluronic acid, TIMP-1 and PIIINP) displayed a high accuracy in predicting fibrosis, although its use may be limited by cost and local availability[105]. Cytokeratin-18 (CK-18) is a serological marker of apoptosis that can be used alone or in combination and was highly accurate for

NASH detection[106]. Its main limitation for clinical use is that a well-established cutoff is not yet defined.

So far, studies evaluating the different serum biomarkers are comprised of general patients with NAFLD and there is no specific test for patients with DM. Recently, we studied several serum biomarkers in 78 biopsy-proven NAFLD diabetic patients and showed an association between low levels of adiponectin and TGF-β1 with severe NAFLD stages[67]. Maybe the combination of two methods like serum biomarkers and imaging methods might be the best tool for predicting NASH and advanced fibrosis.

The histological diagnosis of NAFLD is defined as the presence of lipid deposit in more than 5% of the hepatocytes independent of the localization into the hepatic lobule. However, the most important issue is the definition of NASH, owing to its prognostic value. Brunt *et al*[16] in 1999 classified NAFLD into three different stages: mild, moderate and severe. Likewise, Matteoni's classification of NAFLD was based on the severity of hepatic lesion as follows: type 1, isolated steatosis; type 2, steatosis and lobular inflammation; type 3, steatosis and ballonization of hepatocytes; and type 4, which added the presence of hyaline bodies and fibrosis to the previous stages. Stages 3 and 4 were considered as NASH[15]. Kleiner *et al*[17] in 2005 proposed an update on Brunt's classification and defined a score named NAS, based on the sum of three criteria: steatosis (graded 0 to 3), lobular inflammation (graded 0 to 3) and ballonization (graded 0 to 2). A NAS $\geq$ 5 points implies an advanced inflammatory activity. However, this score should not be applied to diagnose NASH because many patients have NASH with a NAS < 4 points. The NAS is a useful tool to evaluate treatment response and should be used in this situation. Thus, the hallmarks to the diagnosis of NASH are the histological findings observed in liver biopsy and not its intensity. Currently, NASH is defined by the combination of steatosis and necroinflammatory lesions, like ballonization, with or without fibrosis[107].

TREATMENT

There are very few randomized, blinded and controlled clinical trials of drugs with sufficient duration and adequate histological outcomes in patients with NAFLD and DM. Hence, data on treatment of NAFLD in diabetic patients are scarce, and treatment of NAFLD in diabetic patients is conducted based on evidences from mixed populations of diabetic and non-diabetic individuals.

Weight loss following caloric restriction and physical exercise improves insulin sensitivity and cardiometabolic risk factors. However, both implementation and maintenance of these lifestyle interventions pose challenges for most of the individuals[108,109]. A 5% weight loss through lifestyle modification improved liver biochemistry and reduced hepatic steatosis[110], however at least a 10% weight reduction was required for a significant improvement in inflammation, ballooning, and NAS[110]. Notably, seden-

Ref.	Sample size	Type 2 diabetes	Type of intervention	Study design/ duration	Liver enzymes	Imaging	Histology
Kistler *et al*[111]	813 adults	25%	Inactive or moderate or vigorous exercise	Retrospective analysis of biopsy-proven NAFLD	Vigorous recommendations was associated with ↓ GGT levels compared with being inactive		Vigorous exercise was associated with a ↓ adjusted odds of NASH
Hallsworth *et al*[112]	19 adults		Resistance exercise	Randomly assigned to either exercise or standard care. 8 wk	No significant changes in ALT levels	Resistance exercise: 13% relative ↓ in liver lipid by ¹H-MRS	
Bacchi *et al*[113]	31 adults	100%	Aerobic (AER) or resistance (RES) training	Randomized controlled study. 4 mo		Hepatic fat content was ↓ in both by in-opposed-phase MR imaging	
Mathurin *et al*[114]	381 adults	25%	Bariatric surgery	Prospective study. Follow-up of 5 yr	Significant ↓ in ALT and GGT levels 1 and 5 yr after bariatric surgery		Significant ↓ in NASH Fibrosis ↑, 96% with F1
Mummadi *et al*[115]	766 paired liver biopsies		Bariatric surgery	Systematic review and meta-analysis (15 studies)			↓ or resolution 81.3% in NASH and 65.5% in fibrosis

Table 2 Current data on non-pharmacological treatments of non-alcoholic fatty liver disease

↓: Decrease; ↑: Increase. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gammaglutamyl transferase; AP: Alkaline phosphatase; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; ¹H-MRS: Proton magnetic resonance spectroscopy; F1: Stage 1 of fibrosis.

tary patients with NAFLD and DM should undergo a cardiovascular risk assessment before initiating a fitness program, especially before a high intensive training. In a retrospective study with 813 individuals with biopsy-proven NAFLD from the Nonalcoholic Steatohepatitis Clinical Research Network, neither moderate intensity exercise nor total exercise per week were associated with NASH or stage of fibrosis. In this study, meeting vigorous recommendations was associated with decreased adjusted odds of having NASH. This study suggested that maybe the intensity of the exercise could be more important than its duration[111]. Notwithstanding, Hallsworth *et al*[112] showed that independent of weight loss, moderate anaerobic exercise seemed to improve insulin sensitivity and hepatic steatosis. In a recent randomized controlled trial, Bacchi *et al*[113] compared the effects of aerobic (AER) or resistance (RES) training on hepatic fat content in 31 type 2 diabetic subjects with NAFLD. Hepatic fat content was markedly reduced in both AER and RES training groups. In addition, hepatic steatosis, defined as hepatic fat content > 5.56% by an in-opposed-phase magnetic resonance imaging technique, was not detected in about one-quarter of the patients in each intervention group.

Because NAFLD is present in the majority of patients who undergo bariatric surgery, there has been growing interest in evaluating the role of foregut surgery in NAFLD treatment. In a prospective study with 381 severely obese adults followed-up for 5 years after surgery, significant improvements in steatosis, ballooning, NAS and resolution of NASH were observed, changes already present at the first year[114]. After 5 years, levels of fibrosis increased, but 95.7% of patients maintained a grade 1 fibrosis. As none of the patients had advanced fibrosis at entry, the effect of bariatric surgery on liver fibrosis could

not be evaluated[114]. In a meta-analysis that evaluated the influence of bariatric surgery on liver histology in adults with NAFLD, Mummadi *et al*[115] found that steatosis, NASH, and fibrosis improved or completely resolved in a significant proportion of patients. At this moment, there is still no clear evidence indicating foregut bariatric surgery as an established option to specifically treat NASH, but it may provide benefit in NAFLD treatment in otherwise eligible obese individuals[115]. Table 2 summarizes the principal studies concerning non-pharmacological interventions in patients with NAFLD.

High doses of omega-3 polyunsaturated fatty acids (PUFAs) are effective in treating hypertriglyceridemia, which is often a feature of NAFLD and T2DM. The efficacy of omega-3 PUFAs supplementation in NAFLD has recently been examined in a systematic review of nine eligible studies, involving 355 patients with NAFLD[116]. This systematic review with different doses of omega-3 PUFAs demonstrated significant reductions in hepatic fat content. However, at this point, the optimal dose and duration of this therapy is not yet established. A large randomized placebo-controlled trial of two doses of eicosapentanoic acid is under way in the United States.

Many drugs have been evaluated in NAFLD management. The main studies on pharmacological treatments of NAFLD are resumed on Table 3. Statin therapy is recommended in patients with overt cardiovascular disease and in almost all patients with T2DM. Additionally, these drugs can be used in dyslipidemic subjects with increased baseline liver enzymes and may even produce some histological benefit in NASH[117,118]. Ursodesoxycholic acid (UDCA) is a secondary bile acid with lipid lowering, anti-apoptotic and anti-inflammatory properties. There has been initial interest in the use of UDCA to treat NAFLD, although double-blind, randomized, placebo-controlled

Table 3 Current data on pharmacological treatments of non-alcoholic fatty liver disease

Ref.	Sample size	Type 2 diabetes	Type of intervention/ drug	Study design/ duration	Liver enzymes	Imaging	Histology
Parker *et al*[116]	355		Omega-3 PUFA: 0.8-13.7 g/d	Systematic review and meta-analysis (9 studies) median duration of treatment: 6 mo	Significant efficacy of PUFA on ALT and AST levels	Significant efficacy of PUFA on liver fat (US, ^{1}H-MRS)	
Lindor *et al*[119]	166		UDCA: 13-15 mg/kg per day	Randomized placebo-controlled study (24 mo)	No significant changes in ALT, AST and GGT levels with UDCA		No significant changes in NASH or fibrosis with UDCA
Leuschner *et al*[120]	185		UDCA: 23-28 mg/kg per day	Randomized placebo-controlled study (18 mo)	No significant changes in ALT and AST, ↓ GGT levels with UDCA		No significant changes in NASH or fibrosis with UDCA
Uygun *et al*[121]	36	0%	Metformin: 1.7 g/d	Randomized placebo-controlled study (6 mo)	Significant ↓ in ALT and AST levels with metformin	Significant efficacy of metformin on liver fat (US)	No significant ↓ in inflammatory activity or fibrosis with metformin
Haukeland *et al*[122]	48	27%	Metformin: 2.5-3 g/d	Randomized placebo-controlled study (6 mo)	No significant changes in ALT, AST levels with metformin	No significant ↓ on liver fat (CT) with metformin	No significant changes in NASH with metformin
Shields *et al*[123]	19	0%	Metformin: 500 mg-1 g/d	Randomized placebo-controlled trial (12 mo)	No significant changes in ALT and AST levels with metformin		No significant changes in NASH or fibrosis with metformin
Lutchman *et al*[126]	18	0%	Pioglitazone: 30 mg/d	Prospective open study (12 mo)	ALT levels normalized in 72%	Hepatic fat content was ↓ by MR imaging	significant ↓ in necroinflammation and fibrosis with pioglitazone
Belfort *et al*[127]	55	48%	Pioglitazone: 45 mg/d	Randomized placebo-controlled study (6 mo)	Significant efficacy of pioglitazone on ALT and AST levels	Significant efficacy of pioglitazone on liver fat (^{1}H-MRS)	Significant ↓ in necroinflammation but not in fibrosis with pioglitazone
Aithal *et al*[128]	74	0%	Pioglitazone: 30 mg/d	Randomized placebo-controlled trial (12 mo)	Significant ↓ in ALT and GGT levels		Significant ↓ in inflammatory activity and fibrosis with pioglitazone
Sanyal *et al*[129]	247	0%	Vitamin E: 800 UI/d Pioglitazone: 30 mg/d	Randomized placebo-controlled trial (24 mo)	Significant ↓ in ALT, AST and GGT levels with both treatments		Significant ↓ of NASH with vitamin E. No changes in fibrosis with either treatment

↓: Decrease; ↑: Increase. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gammaglutamyl transferase; AP: Alkaline phosphatase; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; PUFA: Polyunsaturated fatty acids; UDCA: Ursodeoxycholic acid; US: Ultrasonography; MR: Magnetic resonance; ^{1}H-MRS: Proton magnetic resonance spectroscopy; CT: Computed tomography.

trials with doses ranging from 13 to 28 mg/kg per day and pre- and post-treatment liver biopsies have yielded disappointing results[119,120].

Given the importance of insulin resistance in the pathogenesis of NAFLD, insulin-sensitizing agents have been investigated in the treatment of this condition in patients with and without diabetes. Metformin reduces endogenous glucose production and improves whole-body insulin sensitivity. It is the first-line choice in oral therapy for patients with T2DM. Metformin has beneficial effects on serum aminotransferases and insulin resistance. However, in patients with NAFLD without T2DM, a number of small randomized placebo-controlled clinical trials with different doses (1500-2000 mg/d) and short durations (6-12 mo) have failed to demonstrate an improvement in liver steatosis, inflammation or fibrosis[121-123]. In spite of these poor results, there is evidence from case-control and population-based studies that the

use of metformin was associated with risk reduction for the development of hepatocellular carcinoma in diabetic patients[124,125].

Rosiglitazone and pioglitazone are peroxisome proliferator-activated receptor γ (PPAR γ) agonists that redistribute fat from the muscle and liver to peripheral adipose tissue and, thereby, improve insulin resistance. Concerns have been raised regarding an association between increased cardiovascular risk with rosiglitazone and its use has been restricted. Three studies of pioglitazone with doses ranging from 30 to 45 mg found a significant improvement in liver histology when compared with placebo in patients with NASH[126-128], but improvement of fibrosis was demonstrated in only one study[128]. Moreover, among these studies only one examined a cohort of patients with T2DM or impaired glucose tolerance with NASH; in this study, pioglitazone significantly improved steatosis, hepatocellular ballooning, inflammation and

necroinflammation, compared with placebo. Improvement in NAS was seen in 73% of patients treated with pioglitazone compared to 24% of placebo-treated patients, and there was a trend toward improvement in fibrosis in patients receiving pioglitazone[127]. The PIVENS study[129] is a recent clinical trial that randomized 247 non-diabetic patients with biopsy-proven NASH to pioglitazone 30 mg/d, vitamin E 800 IU/d, or placebo for 24 mo. The primary outcome was histological improvement in the features of NASH. Pioglitazone, as compared to placebo, was not associated with a significantly higher rate of improvement in the composite NAS score. However, both vitamin E and pioglitazone treatment improved the scores of steatosis, inflammation, ballooning, and serum aminotransferase levels[129]. It seems that liver histology benefits obtained with pioglitazone therapy may disappear with its discontinuation. Nonetheless, there is a debate surrounding the long-term risk-benefit ratio of pioglitazone therapy. The most frequent side-effects of pioglitazone are weight gain of 2-5 kg and bone loss with fractures[130]. Pioglitazone treatment can also precipitate congestive heart failure in patients with preexisting cardiac failure[131]. In addition, increased bladder cancer risk has been recently associated with pioglitazone use in diabetic patients[132].

Glucagon-like peptide-1 (GLP-1) agonists and dipeptidyl peptidase-IV (DPP-IV) inhibitors are new pharmacological agents with multiple anti-hyperglycemic actions. The biological activities of GLP-1 agonists include glucose-dependent insulin secretion, suppression of postprandial glucagon to reduce hepatic glucose release and slowing of gastric emptying. There is also evidence that GLP-1 agonists have beneficial effects on the liver, including suppression of hepatic lipogenesis and stimulation of lipid oxidation[133,134]. A recent meta-analysis of two GLP-1 agonists, liraglutide and exenatide, in populations with and without diabetes, including data on liver enzyme tests from 12 of the 25 trials included, found that ALT concentrations decreased after treatment with the liraglutide but not with exenatide[135]. Clinical trials with these agents in patients with NAFLD with or without T2DM are ongoing and their results are awaited in the next years.

Increased oxidative stress occurs in NAFLD and T2DM. Among antioxidant compounds, vitamin E has the most significant evidence supporting its use. In the PIVENS study[129], vitamin E supplementation, 800 UI/d, resulted in significant improvement in pathological features of NASH. The improvement in NAS was observed in 42% of patients receiving vitamin E compared with 19% of patients receiving placebo. Nevertheless, caution must be applied regarding the long-term safety of vitamin E, especially in doses greater than 400 IU/d, which may be associated with increased risk of all-cause mortality[136]. Currently, there is no evidence regarding vitamin E effectiveness and safety in diabetic patients with NASH or in patients with NASH-related cirrhosis.

GFT505, a dual peroxisome proliferator-activated receptor (PPAR)-α/δ agonist, improved peripheral and hepatic insulin sensitivity in a randomized crossover study to subsequent 8-week treatment periods with GFT505 (80 mg/d) or placebo. GFT505 also reduced liver enzyme concentrations and could be a promising drug candidate for the treatment of T2DM and NAFLD. There was no indication of PPARγ activation and no safety concern with GFT505[137].

Obeticholic acid (OCA), a farnesoid X agonist receptor, is a semi-synthetic human bile acid that regulates glucose and lipid metabolism. Data from a small pilot study demonstrate that OCA improves insulin sensitivity compared with placebo. Also, of importance, OCA appears to improve liver injury in patients with T2DM and NAFLD[138]. Larger studies with longer duration of therapy and follow-up are needed to evaluate long-term efficacy of these emerging therapies.

CONCLUSION

Patients with DM and NAFLD are prone to the severest stages of liver diseases and to cardiovascular and liver-related outcomes. The major challenge is to identify these patients by accurate non-invasive methods. Many algorithms and new imaging methods are available but they still need to be validated in this specific population. The ideal treatment would be effective for both NASH and diabetes, but it is not yet available. Given the importance of cardiovascular and liver outcomes in diabetic patients, effective interventions are urgently required in order to prevent progression to these life-threatening and prevalent complications.

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