NONALCOHOLIC FATTY LIVER DISEASE: THE OVERLOOKED COMPLICATION OF TYPE 2 DIABETES

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is a common complication of obesity and type 2 diabetes mellitus (T2DM). Most times it is an unrecognized comorbidity to the primary care provider and endocrinologist. Today it is the most common chronic liver disease in developed countries. It is characterized by insulin resistance and hepatic triglyceride accumulation in the absence of co-existing etiologies, such as excessive alcohol consumption, viral hepatitis, medications or other etiologies for hepatic steatosis. Its more severe form of the disease with steatohepatitis (NASH) is associated with hepatocyte injury (necrosis and inflammation) and frequently with fibrosis. Although it appears to be an indolent condition, with few symptoms and often normal plasma aminotransferases, NASH is a leading cause of end-stage liver disease and hepatocellular carcinoma (HCC), and significantly increases the risk of developing cardiovascular disease (CVD) and T2DM. The pathogenesis of NASH remains poorly understood, and likely to be multifactorial, but insulin-resistant adipose tissue plays an important role. The natural history of NAFLD is incompletely understood, but risk factors for disease progression include weight gain, obesity and T2DM, as well as the severity of fibrosis stage at diagnosis. Diagnostic algorithms are evolving but we offer an approach that integrates for the non-hepatologist plasma biomarkers, imaging, and the role of liver biopsy for the management of these complex patients. At the present time, early screening -with biomarker panels or a liver ultrasound, ideally with transient elastography- is reserved for high-risk patients (i.e., obese patients with T2DM or elevated plasma AST/ALT levels or evidence of steatosis at a random liver exam) until more accurate non-invasive methods are available. A liver biopsy should be considered on a case-by-case basis, to identify those at risk of NASH-cirrhosis, working in close collaboration with a hepatologist. Treatment should include a comprehensive approach with lifestyle modification and therapeutic agents tested in RCTs, such as vitamin E (in patients without diabetes) or pioglitazone for patients with or without diabetes. Pioglitazone, given its low-cost as a generic medication, long-standing track record of efficacy in NASH, and cardiometabolic benefits, is likely to be for NASH what metformin has become for the management of T2DM. However, proper patient selection and close monitoring is needed. In addition, a number of new pharmacological agents are being studied in phase II/III trials and future management will involve the use of combination therapy, as for other chronic metabolic conditions. In summary, endocrinologists need to be aware of the severe metabolic and liver-specific complications of NASH and establish

early-on a long-term management plan. Screening will likely take place in the same way as for diabetic retinopathy or nephropathy. A better understanding of its natural history and pathogenesis of NASH, combined with improved diagnostic and treatment options, will likely place endocrinologists at the forefront of the management efforts to prevent end-stage liver disease in patients with NASH.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver condition that is on the rise. It has become the most common chronic liver condition in many parts of the world. It encompasses a wide spectrum of disease with different clinical implications. NAFLD means that there is evidence of liver steatosis, either by imaging or histology, in the absence of secondary causes of hepatic fat accumulation such as significant alcohol consumption, chronic use of steatogenic medication, or another established chronic liver disease. Between 40 to 50% of patients that are obese have NAFLD and this rises to about 70% if they have type 2 diabetes mellitus (T2DM). In its simplest form, known as isolated steatosis or NAFL (nonalcoholic fatty liver), there is triglyceride accumulation of ≥5% without evidence of hepatocellular injury in the form of hepatocyte ballooning or evidence of fibrosis. Although the natural history of this condition remains uncertain, and may possibly progress to more severe disease, at the present moment NAFL is considered to be associated with limited risk of liver morbidity. However, it is associated with insulin resistance so that the liver can be seen as a "mirror" of metabolic health (i.e., in obesity steatosis being a reflection of insulin resistance, and in particular, of adipose tissue dysfunction) and with an increased risk of cardiovascular disease (CVD). In its more severe form, known as nonalcoholic steatohepatitis or NASH, steatosis ≥5% is associated with hepatocyte injury with necrosis ("ballooning") and lobular inflammation, with or without fibrosis.

Steatohepatitis is often a progressive disorder in T2DM associated with the development of fibrosis that can eventually lead to cirrhosis. Liver fibrosis is defined by its severity in stages ranging from absence of fibrosis (stage F0) to mild (stage F1), moderate (stage F2, with zone 3 sinusoidal fibrosis plus periportal fibrosis), and "advanced" fibrosis referring specifically to stages 3 (bridging fibrosis) or 4 (cirrhosis). Having fibrosis is the most important histological feature of NAFLD associated with long-term

mortality. Fibrosis also predisposes patients to hepatocellular carcinoma (HCC).

Type 2 diabetes mellitus has been a well-established factor in the progression of NAFLD to more severe forms, including a higher incidence of HCC (1-3). However, in clinical practice NAFLD still remains an under-recognized complication of T2DM, unlike the other microvascular and macrovascular complications.

As discussed later, isolated steatosis and NASH may carry an increased risk of CVD and being the most common cause of death in patients with NAFLD, independent of other metabolic comorbidities. It is important for endocrinologists and primary care physicians to recognize that NAFLD in T2DM has been shown to be associated with adverse metabolic changes resulting in increased atherosclerotic disease and cardiovascular consequences (4,5).

NAFLD: KEY CONCEPTS

The incidence of NAFLD is rising, paralleling that of obesity and diabetes mellitus. There has been extensive research in the area of NAFLD, especially over the past two decades. However, given the lack of highly reliable noninvasive diagnostic methods, the burden of NAFLD probably remains overlooked. By liver ultrasound, studies have demonstrated the prevalence of NAFLD to be 24% in United States, whereas using blood testing alone this is underestimated at just 13% (6). By the gold-standard magnetic resonance imaging and spectroscopy (^{1}H-MRS), the prevalence of NAFLD in the general population is estimated to be 34% (7).

Unfortunately, imaging techniques cannot adequately evaluate for hepatocellular necrosis or inflammation (i.e. NASH). Studies that have utilized a liver biopsy to confirm the diagnosis of NAFLD have shown that 59% of patients with NAFLD have NASH, this being much higher in obese individuals (6). Moreover, recent studies have reported that about 18% of unselected patients with T2DM have moderate-to-severe (F2-F4) fibrosis (6,8).

NAFLD often progresses to steatohepatitis (NASH), especially in patients with T2DM. NASH is hallmarked by hepatocellular necrosis, lobular inflammation and often fibrosis. Many studies have now documented that patients with NASH and fibrosis have the worst mortality (9). As

fibrosis progresses, cirrhosis develops. This rate of progression to cirrhosis is highly variable and dependent on age, BMI, blood pressure control, presence of T2DM, and degree of steatohepatitis (10). The three most relevant risk factors are obesity (excessive BMI or visceral obesity), T2DM, and presence of moderate to severe fibrosis (11). However, given the high heterogeneity in disease progression one must admit that the precise factors leading to cirrhosis remain unclear.

NASH is currently the second most common indication for liver transplantation, after hepatitis C. It is predicted to be the leading indication for liver transplantation in the next decade given the rise in incidence (8). The annual incidence of HCC in NAFLD – related cirrhosis is about 1% (1,8,12,13). Nonalcoholic steatohepatitis related cirrhosis is currently the third leading cause for HCC, after HCV and alcohol-related liver disease. Importantly, those with NASH-related HCC that undergo liver transplantation are more likely to have a higher BMI and higher rate of T2DM (13). In one study, it has also been demonstrated that HCC can develop in NASH in the absence of cirrhosis (14).

NAFLD and Cardiovascular Disease

Many factors lead to cardiovascular disease in patients with T2DM and NAFLD. For instance, they have increased intrahepatic triglyceride accumulation and insulin resistance. This is associated with increased hepatic VLDL secretion and a decrease in the peripheral clearance of triglyceride-rich lipoproteins. This results in a proatherogenic profile, which includes hypertriglyceridemia, low HDL-C, and an increase in small, dense LDL particles, plus a state of subclinical inflammation (8).

These patients also often have more severe hepatic insulin resistance leading to progressive deterioration of glycemic control (9). Hepatic insulin resistance is associated with

hyperinsulinemia from increased insulin secretion and decreased insulin clearance (3,15). Hyperinsulinemia *per se* has been associated with atherogenesis in animal models of disease and in epidemiological studies. Chronic hyperinsulinemia also causes downregulation of insulin signaling pathways and acquired insulin resistance in short-term clinical studies in humans (11). In this context, hyperglycemia is more severe and also appears to contribute to CVD. Endothelial dysfunction also has been shown to cause increased cardiovascular risk in patients with NAFLD (16). Early left ventricular "diastolic dysfunction" (or heart failure with preserved ejection fraction or HFpEF) has been noted in patients with NAFLD and well controlled T2DM independent of other risk factors (17). Patients with NAFLD are often found to have a significantly worse carotid intima-media thickness with increased atherosclerotic disease when compared with clinically matched patients without NAFLD. This has been correlated in some studies with an advancing degree of steatosis, inflammation, and/or fibrosis (18,19). In NASH with cirrhosis, CVD is the leading cause of mortality (1,8,20).

Thus, it is not unexpected that in NAFLD many studies have reported a higher rate of CVD (Tables 1 and 2). In addition to insulin resistance and hyperinsulinemia, NAFLD and CVD cluster with other common risk factors, including hypertension, hyperlipidemia, T2DM, obesity and inflammation (8). The evidence of the association between NAFLD and increased CVD often persists even after adjusting for traditional cardiovascular risk factors (Tables 1 and 2) (9,21). This suggests that the presence of NAFLD may independently increase an individual's cardiovascular risk, but whether this is worse in patients with steatohepatitis compared to those just having isolated steatosis remains controversial. It should also be noted that many investigators have failed to see the association of NAFLD with CVD after adjusting for traditional cardiovascular risk factors, as detailed in Tables 1 and 2.

Table 1. Cardiovascular Disease in Patients with NAFLD without Type 2 Diabetes						
Author (year)	NAFLD vs controls (n)	Diagnosis of NAFLD	Primary endpoint	Increased CVD	Adjusted CV risk	Study design
Villanova et al (22)	80	Liver biopsy	Endothelial function	Yes	Yes	Prospective case-control, Cross-sectional

Brea et al (23)	80	Ultrasound (US)	Carotid intima-media thickness test (CIMT)	Yes	No	Case-control, Cross-sectional
Adams et al (24)	420	US, CT, MRI or Liver biopsy	All cause and CV mortality	Yes	No	Retrospective cohort, Longitudinal
Volzke et al (25)	4222	Ultrasound	CIMT	Yes	No	Case-control, Cross-sectional
Ekstedt et al (26)	129	Liver biopsy	All cause and CV mortality	Yes [*]	Yes	Retrospective cohort, Longitudinal
Mirbagheri et al (27)	171	Ultrasound	Coronary angiography	Yes	Yes	Cross-sectional
Hamaguchi et al (28)	1221	Ultrasound	CV events	Yes	Yes	Prospective cohort, Longitudinal
Schindhelm et al (29)	1439	ALT	CV events	Yes	Yes	Retrospective cohort, Longitudinal
Fracanzani et al (30)	375	Ultrasound	CIMT	Yes	Yes	Case-control, Cross-sectional
Goessling et al (31)	2812	AST, ALT	CV events	Yes	No	Retrospective cohort, Longitudinal
Aygun et al (32)	80	Liver biopsy	CIMT	Yes	Yes	Prospective case-control, cross-sectional
Haring et al (33)	4160	Ultrasound	All cause and CV mortality	Yes**	Yes	Retrospective cohort, Longitudinal
Rafiq et al (34)	173	Liver biopsy	All cause and CV mortality	No***	No	Retrospective cohort, Longitudinal
Salvi et al (35)	220	Ultrasound	Arterial stiffness by carotid-femoral pulse wave velocity	Yes	Yes	Case-control, Cross-sectional
Soderberg et al (36)	118	Liver biopsy	All cause and CV mortality	Yes	Yes	Retrospective cohort, Longitudinal

Zhou et al (37)	3324	Ultrasound	All cause and CV mortality	Yes	Yes	Retrospective cohort, Longitudinal
Stepanova et al (38)	11,613	Ultrasound	All cause and CV mortality	Yes	No	Retrospective cohort, Longitudinal
Lee et al (39)	1442	Ultrasound	Arterial stiffness by brachial-ankle pulse wave velocity	Yes	Yes	Case-control, Cross-sectional
Kozakova et al (40)	1012	Fatty Liver Index	CIMT	Yes	Yes	Cross-sectional
Kim et al (41)	4023	Ultrasound	Coronary artery calcification score by CT	Yes	Yes	Cross-sectional
Hallsworth et al (42)	38	MR spectroscopy	LV dysfunction by cardiac MRI	Yes	Yes	Case-control, Cross-sectional
Colak et al (43)	72	Liver biopsy	Endothelial function by flow mediated dilation (FMD) and CIMT	Yes	Yes	Observational case-control, cross-sectional
Pisto et al (44)	988	Ultrasound	CV events	Yes	No	Retrospective cohort, Longitudinal
Ekstedt et al (45)	2515	Liver biopsy	CV events	Yes	No****	Retrospective cohort, Longitudinal
Zeb et al (46)	4119	CT	CV events	Yes	Yes	Prospective cohort, Longitudinal
Fracanzani et al (47)	273	Ultrasound	CIMT	Yes	Yes	Prospective cohort, Longitudinal
Wong et al (48)	612	Ultrasound	CV events, Coronary artery stenosis by angiogram	Yes*****	No	Prospective cohort, Longitudinal

*Patients with NASH but not with only steatosis had increased cardiovascular mortality.

** NAFLD was associated with increased all cause and cardiovascular mortality in men only.

***Compared NASH vs non-NASH NAFLD patients, no difference in overall mortality was found, but liver mortality was significantly different, with higher rates in NASH patients. Overall, most common causes of death reported were cardiovascular disease, malignancy and liver related deaths.

****No increased CV risk when diabetics were excluded.

***** Patients with NAFLD were more likely to have significant coronary artery stenosis at baseline, and more likely to undergo percutaneous coronary intervention; however, no increased association of NAFLD with CV events during follow up.

Table 2. Cardiovascular Disease in Patients with NAFLD with Type 2 Diabetes						
Author	NAFLD vs controls (n)	Diagnosis of NAFLD	Primary Endpoint	Increased CVD	Adjusted CV Risk	Study Design
Targher et al (49)	200	Ultrasound	CIMT	Yes	Yes*	Cross-sectional
Targher et al (50)	2103	Ultrasound	CV events	Yes	Yes	Longitudinal
McKimmie et al (51)	623	CT	CIMT and coronary artery calcium score	No	No	Cross-sectional
Petit et al (52)	101	MR spectroscopy	CIMT	No	No	Prospective, Cross-sectional
Adams et al (53)	337	Liver US, CT or biopsy	All-cause mortality and CVD	No	No	Longitudinal
Poanta et al (54)	56	Ultrasound	CIMT	No	No	Case-control, Cross-sectional
Bonapace et al (55)	50	Ultrasound	LV diastolic dysfunction	Yes	Yes	Cross-sectional, Prospective
Dunn et al (56)	2343	CT	CV mortaility	No	No	Retrospective cohort, Longitudinal
Khashper et al (57)	93	CT	Coronary artery calcium score	No	No	Prospective, Cross-sectional
Kim et al (58)	4437	Ultrasound	CIMT	Yes	Yes	Cross-sectional
Idilman et al (59)	273	CT	Coronary artery	Yes**	Yes	Prospective, Cross-sectional

			calcium score			
Silaghi et al (60)	336	Ultrasound	CIMT	No	No	Cross-sectional
Kwak et al (61)	213	Ultrasound	Coronary artery calcium score	Yes***	Yes	Cross-sectional
Mantovani et al (62)	222	Ultrasound	LV diastolic dysfunction	Yes	Yes	Cross-sectional

*CV risk remained significant after adjustment for other traditional cardiovascular risk factors, however did not remain significant after adjustment for HOMA-IR.
**Only significant association was between NAFLD and significant CAD (defined as more than or equal to 50% stenosis in at least one coronary artery).
***Only significant association in patients with NAFLD and A1C > 7% but not in lower A1C.

NAFLD and Chronic Kidney Disease

The presence of NAFLD and NASH with fibrosis have been recently associated with chronic kidney disease (CKD), and more severe forms of fatty liver disease correlate with worse and progressive stages of CKD. In most studies, CKD has been defined as having an estimated glomerular filtration rate (eGFR) < 60 ml/min/1.73m^2 or increased albuminuria/proteinuria (20,63,64). In a case control study by Targher et al, the severity of liver histology in patients with biopsy-proven NASH was found to be independently associated with the degree of worsening eGFR (65).

A cross-sectional study of Japanese patients with biopsy-proven NAFLD showed an increased prevalence of CKD with worsening liver histology. They found that overall, 14% of patients with NAFLD had evidence of CKD. Of the patients with biopsy proven NASH, 21% had the presence of CKD; and of the patients with NAFLD with no evidence of NASH, only 6% had CKD (64). This was higher than in patients without NAFLD or NASH. The pathophysiology of this association is not well understood, but the increased atherogenicity associated with NAFLD is likely a contributing factor (20). A more recent meta-analysis also showed a higher prevalence of CKD in patients with NASH when compared with patients with NAFLD without NASH, and a higher prevalence of CKD in patients with advanced fibrosis when compared with patients with lower degree of fibrosis (63).

NAFLD and Polycystic Ovarian Syndrome

Women with polycystic ovarian syndrome (PCOS) have been found to have an increased prevalence of NAFLD. This association has been present even after adjusting for other factors associated with the metabolic syndrome, such as BMI, hypertension, and type 2 diabetes mellitus (66,67). Evidence of hyperandrogenism, especially with testosterone level > 3 nmol/L has been associated with increased risk of NAFLD in women with PCOS (66,68).

PATHOGENESIS

Of note, the pathogenesis of NASH is poorly understood in humans. Most proposed mechanisms at the molecular level have only been observed in cell systems or animal models, but not confirmed in humans. Animal models of NASH are far from ideal in resembling human disease (69). Often treatments that are promising in animal models are in discordance with results in humans – indeed, most treatments that have resolved NASH, and even fibrosis, in mice have failed so far in large RCTs. A detailed description of the potential pathways leading to steatohepatitis exceeds the scope of this review, therefore we refer the reader to recent in-depth reviews involving a broad spectrum of mechanisms involved in the development of NASH and liver fibrosis (11,69-72). In **Figure 1** (below) we propose a schematic representation of the factors and many pathways leading to NASH and fibrosis.
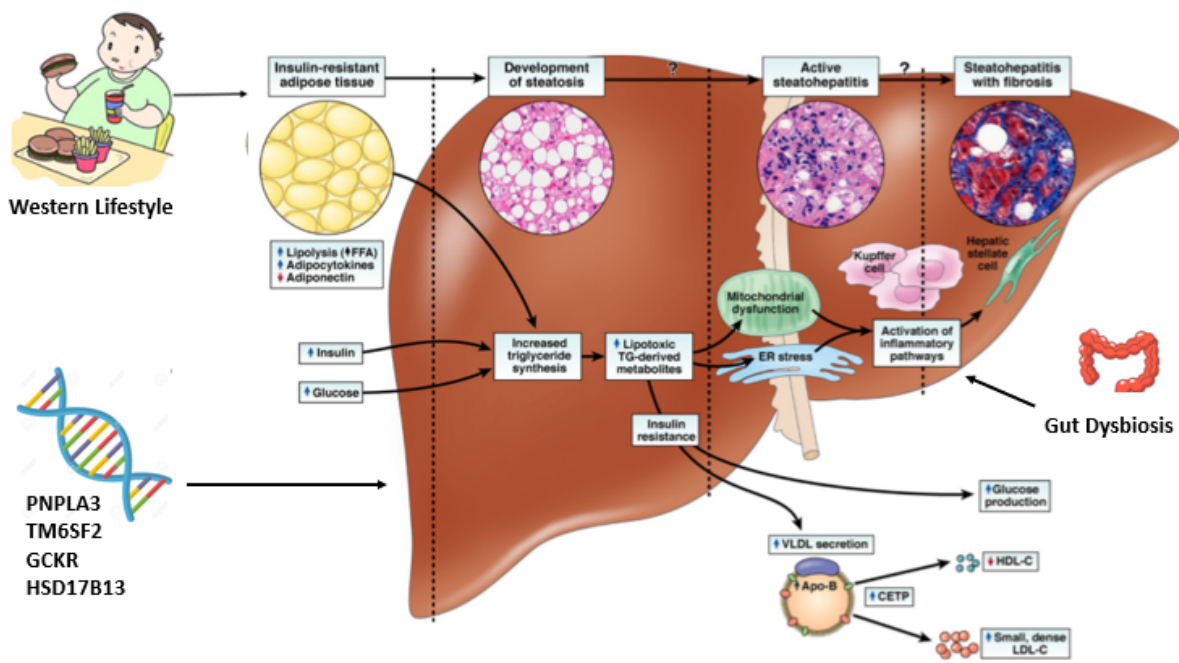
Figure 1: Pathogenies of NAFLD, adapted from Cusi K (11).
PNPLA3=patatin-like phospholipase domain-containing protein 3. TM6SF2=transmembrane-6 superfamily member 2. GCKR=glucokinase regulator. HSD17B13=hydroxysteroid 17-beta dehydrogenase 13. NAFLD=non-alcoholic fatty liver disease. HDL-C=high-density lipoprotein cholesterol. LDL-C=low-density lipoprotein cholesterol. VLDL=very low-density lipoprotein. CETP=Cholesteryl ester transfer protein.

Development of Steatosis

Clinical studies have shown that the source of intrahepatic triglycerides in NAFLD is about two-thirds from free fatty acids originating from adipose tissue. However, higher rates of *de novo* lipogenesis (DNL) are also observed in obesity and T2DM (73). In obesity, adipocytes adapt to chronic excess energy supply by undergoing hypertrophy and hyperplasia. This is likely a protective adaptation to allow for an increase in adipocyte storage capacity and ameliorate the potential for ectopic triglyceride accumulation in tissues with limited ability to do so such as the liver, skeletal muscle, pancreas and others. When these adaptive mechanisms are overwhelmed by a chronic excess in nutrient supply, the chronic flux of FFAs promotes a state of "lipotoxicity" across different tissues (11). Adaptation to chronic overnutrition occurs at the expense of developing adipose tissue insulin resistance and triggering mechanisms that attract macrophage accumulation and activation in fat and systemic subclinical inflammation. Moreover, it has been shown that hypertrophic adipocytes share a gene expression pattern that is similar to macrophages and produce adipocytokines similar to those produces by foam cells (74). Adipocytokines have a key role to play in the pathogenesis of insulin resistance by inhibiting insulin signaling pathways via action of insulin receptor substrate (IRS)-1 and c-Jun N terminal kinase (JNK) pathways. Insulin resistance and inflammation is also triggered by the generation of reactive oxygen species, and lipid intermediates such as diacylglycerol (DAG) (75), ceramides (76, 77) and acylcarnitines (77).

Normally, insulin decreases gluconeogenesis and increases hepatic synthesis of
fatty acids and triglycerides. Based on animal models of T2DM, it has been postulated that there may be a selective hepatic insulin resistance to glucose metabolism pathways (i.e., inhibition of gluconeogenesis) while preservation of insulin sensitivity at lipid synthetic pathways (78, 79). Selective insulin resistance in the gluconeogenic pathway would explain (at least in part) how hyperinsulinemia may attempt to normalize glucose metabolism at the expense of driving triglyceride synthesis, as hepatic lipid synthetic pathways retain a normal insulin sensitivity, explaining the

etiology of both hyperglycemia and hypertriglyceridemia in diabetes. More recently, Perry et al (80) reported that the major mechanism by which insulin suppresses hepatic glucose production appears to be through a reduction in hepatic acetyl CoA by suppression of lipolysis in white adipose tissue (WAT). This is associated with a reduction in pyruvate carboxylase flux. Of interest, insulin's ability to inhibit hepatic acetyl CoA and lipolysis is lost in high-fat-fed rats, a phenomenon reversible by IL-6 neutralization and inducible by IL-6 infusion (80).

However, the above relationship between hyperinsulinemia and steatosis does not completely explain the role of both factors in patients with NASH. In subjects with hepatic steatosis, increasing insulin levels only have a modest correlation with the severity of intrahepatic triglyceride accumulation (81) and there is no relationship between hyperinsulinemia or hepatic steatosis with the severity of inflammation, hepatocyte ballooning (injury), or fibrosis (15, 81).This is despite patients with NASH having worse hyperinsulinemia compared to patients with isolated steatosis (NAFL). This suggests that other mechanisms play a role in human disease.

Lipotoxicity has been extensively studied in skeletal muscle, where accumulation of ectopic triglycerides promotes the formation of toxic lipid metabolites (i.e., such as DAGs) that are closely associated with impairment in insulin signaling. Lipid infusions in healthy subjects have shown that at levels of plasma FFAs typically seen in obesity and NAFLD, there is suppression of insulin signaling and hence development of insulin resistance (82). Lipotoxicity has also reported in pancreatic beta-cells in humans. Normally, FFAs are the main energy source in the fasting state, with a switch to using glucose as the primary fuel after a meal. However, chronically elevated plasma FFA concentrations impair insulin secretion in subjects that are genetically prone to T2DM (83).

NAFLD has been shown to also be a heritable disease (72, 84-90). Nuclear receptors such as peroxisome proliferator-activated nuclear receptors (PPAR) play a key role in hepatic lipid metabolism, however results on association of PPAR and severity of NAFLD have been variable (75). Studies have shown that first-degree relatives of subjects with NAFLD are more susceptible to develop chronic liver disease as compared to the general population (72, 84). Patatin-like phospholipase domain-containing protein 3

(PNPLA3) gene polymorphism has been shown to be associated with worse hepatic steatosis and a worse long-term prognosis in patients with NASH (85). PNPLA3 is usually involved in hydrolysis of hepatocyte triglycerides. This polymorphism results in a loss of function mutation resulting in accumulation of intrahepatic triglycerides. Recently, it has been described that accumulation of PNPLA3 on lipid droplets is the basis of associated hepatic steatosis observed with this polymorphism (86).

Another commonly described polymorphism involves transmembrane 6 superfamily member 2 (TM6SF2) which normally plays a role in interaction between triglycerides and Apolipoprotein B during the extrahepatic secretion of very low-density lipoprotein (87). This polymorphism results in increased hepatic triglyceride deposition, and lower circulating lipoproteins. Recent studies show this polymorphism is associated with higher risk of NAFLD but lower cardiovascular risk (87). A loss of function mutation in the glucokinase regulator (GCKR) gene locus has been implicated in the accumulation of hepatic fat (88,89). Normally, GCKR is involved in controlling the glucose influx into hepatocytes and hence regulating DNL. A protective splice variant HSD17B13 has also been identified. HSD17B13 encodes the lipid droplet protein hydroxysteroid 17-beta dehydrogenase 13 (HSD17B13) (90). This allele was associated with a reduced risk for progression from steatosis to steatohepatitis and fibrosis. Interestingly, it also seems to mitigate the effects of PNPLA3 polymorphism. Finally, an interesting observation is that individuals with familial hypobetalipoproteinemia (FHBL) are prone to NAFL but are characterized by very low levels of plasma low-density lipoprotein (LDL) cholesterol that is protective against CVD (91).

However, at the present time, genetic testing is not recommended in clinical practice as it remains unclear how the presence of a given mutation should modify current management of NASH (92).

## Development of Hepatocyte Injury and Steatohepatitis: Role of Mitochondrial Dysfunction

It should be emphasized that the mechanisms leading to steatohepatitis in humans remain unknown. With limited exceptions that point to subtle defects in mitochondrial function in the liver of subjects with NAFLD and/or T2DM

(reviewed in ref. 71), almost all of the available information has been extrapolated from cell culture studies or animal models of NASH. It is also unclear if NASH is always heralded by isolated steatosis, and what are the drivers of disease. While there is an increasing recognition that NASH is an heterogeneous disease affecting obese and non-obese individuals, disease progression is often with associated with obesity/weight gain and T2DM. Obvious limitations in obtaining sufficient liver tissue for molecular studies, as well as ethical challenges for performing paired liver biopsies before and after a given intervention, have greatly hampered our ability to make significant progress in understanding the pathogenesis of NASH in humans. However, factors associated with overnutrition and insulin resistance likely play a role in the maladaptation of mitochondrial oxidative function that leads to inefficient oxidative flux, accumulation of lipotoxic intermediates and the progression from isolated steatosis to NASH (71,93). As mentioned above, genetic factors may also regulate lipid droplet accumulation that may exacerbate disease progression. Many other trigger factors associated with endoplasmic reticulum (ER) stress, oxidative stress and inflammasome activation have been described. However, the exact temporal relationship and sequence of events remains elusive.

Normally, there is a close regulation between beta-oxidation, hepatic tricarboxylic acid (TCA) cycle activity, ketogenesis and ATP synthesis. Normally, FFAs influx is efficiently dealt through beta-oxidation. However, in states of chronic overfeeding, beta-oxidation can over time become relatively ineffective, resulting in the accumulation of hepatocyte ceramides and DAGs (as well as acylcarnitines), as seen in states of hepatic steatosis (71,75-77). As summarized in Figure 2, the current working hypothesis in NASH is that overactive hepatic TCA cycle carries the risk of overloading the mitochondrial electron transport chain and hence promoting not only the formation of toxic metabolites but the production of reactive oxygen species (ROS) and other inflammatory mediators. In this setting, it is believed that inflammatory pathways are triggered which then lead to hepatocyte necrosis and chronic inflammation, Kupffer cell activation and recruitment, as well as hepatic stellate cell activation. This disruption of the normal equilibrium between hepatocyte and its microenvironment (i.e., in particular with Kupffer cells and hepatic stellate cells, the latter promoting fibrogenesis) seems to determine the degree of hepatocyte injury and the triggering of downstream pathways that lead to cirrhosis, as reviewed in-depth elsewhere (70). However, while many recent interventions successful in animal models have failed in humans, it is of interest that there is a correlation between successful treatment for NASH in humans (with GLP-1RA or pioglitazone [8]) with studies *in vivo* with such interventions that restore hepatocyte TCA function and reduce intracellular toxic lipids (94, 95), giving support to the hypothesis of increased mitochondrial FFA flux as a potential therapeutic target for patients with NASH.
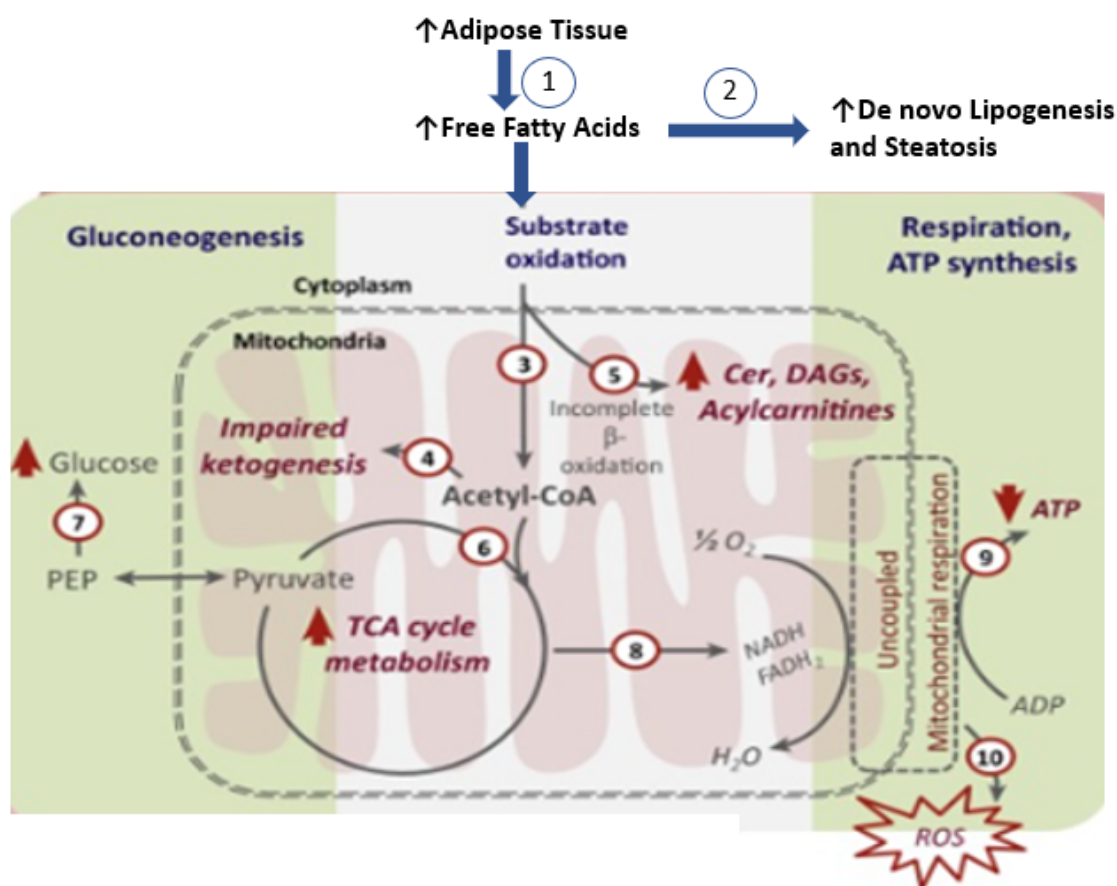
Figure 2. **Hepatic Mitochondrial Oxidative Dysfunction during NASH (71). Adipose tissue insulin resistance results in increased lipolysis and higher flux of FFAs into the liver (1), resulting in high rates of hepatic triglyceride accretion (2). Initial breakdown of FFA in the liver proceeds through b-oxidation, generating two-carbon units of acetyl-CoA (3). During hepatic insulin resistance, disposal of acetylCoA units through ketogenesis undergoes an early compensatory induction in simple steatosis, but is impaired in NASH (4). In spite of FFA overload, hepatic insulin resistance and steatosis result in beta-oxidation being inefficient and incomplete as evident from accumulating levels of hepatic ceramides, DAGs, and long-chain acylcarnitine (5). However, complete oxidation of acetyl-CoA units through the mitochondrial TCA cycle continues unabated during simple steatosis and NASH (6), potentially to meet the energetic demands of maintaining high rates of gluconeogenesis (7). The chronically elevated oxidative flux through TCA cycle during NASH has the potential to uncouple hepatic TCA cycle activity from mitochondrial respiration (8) by disrupting the mitochondrial electrochemical gradient and to impair ATP synthesis (9). This mitochondrial milieu could be a chronic source of ROS generation (10) and cellular inflammation, and could be a target for therapeutic manipulations. Abbreviations: Cer, ceramides; CoA, coenzyme A; DAGs, diacylglycerols; FFAs, free fatty acid; NASH, nonalcoholicsteatohepatitis; PEP, phosphoenolpyruvate; ROS, reactive oxygen species; TCA, tricarboxylic acid.**

However, linking NASH only to altered mitochondrial flux is obviously an oversimplification of a complex web of many factors at play. Other pathways that have been implicated in hepatocyte injury and the development of NASH, although rather broadly, include cholesterol accumulation in hepatocytes (96) and a tangled web involving activation of apoptotic pathways with ER stress and abnormal unfolded protein response (97), as well as defects in autophagy (98). Recently, inflammasome activation has gained attention as it integrates many cytoplasmic signals into danger-associated molecular patterns (DAMPs) from diverse

sources such as intracellular lipids to the gut microbiome (97, 98).

Diet and gut microbiota have been repeatedly implicated to play a role in the pathogenesis of NAFLD. In particular, fructose appears to play a role in NASH by stimulating DNL and suppressing -oxidation of FFAs, hence leading to hepatocyte injury (99). Many studies have shown that excess fructose consumption, usually as sugar-sweetened beverages with sucrose (converted to fructose and glucose after ingestion), is associated with development of NAFLD and NASH. Obesity is also associated with a change in gut microbiota that produce more reactive oxygen species and are involved in triggering a variety of inflammatory pathways (100). However, the causative role of the gut microbiome in the development of T2DM or NAFLD remains overall poorly understood (101).

Development of Liver Fibrosis

Here too the data in humans is scarce and largely limited to *in vitro* and *in vivo* evidence. Potential mechanisms linked to the development of NASH have focused on hepatocyte apoptosis with the release of a broad spectrum of cytokines (e.g., interleukins [-1, -2, -18], hedgehog ligands, TNF- , TGF- , and many others) (11, 97, 98). Wang et al (102) have identified one such pathway (the transcriptional activator TAZ) that appears to play an important profibrogenic role in NASH in a mouse model of NASH. Taken together, this extensive signaling network, triggered by injured hepatocytes, activates nearby Kupffer cells that induce hepatic stellate cells to become myofibroblasts and increase the production of matrix proteins that result in cirrhosis over time. Genetics also appear to play a role as the PNPLA3-I148M variant may not only modify lipid droplet metabolism but have a direct role on stellate cell function in NASH (103). Recently, Lindén et al (104) reported a reduction in liver inflammation and fibrosis in a Pnpla3 knock-in 148M/M mutant mice (with a human PNPLA3 I148M mutation) with a liver-targeted GalNAc3-conjugated antisense oligonucleotide (ASO)-mediated that silenced Pnpla3 expression.

At a clinical level, a recent study examined factors associated with disease progression in a large (n = 475 patients) clinical trial (105). The main factor associated with clinical disease progression is severity of fibrosis at baseline and greater increases in hepatic collagen content, level of alpha-smooth muscle actin, and Enhanced Liver Fibrosis score overtime. Over a follow-up period of 96 weeks, progression occurred in 22% of patients with bridging fibrosis (F3), while liver-related clinical events occurred in 19% of patients with cirrhosis.

Beyond liver histology, from a clinical perspective, practitioners must keep in mind that obesity and T2DM remain the two major risk factors for liver disease progression which calls for screening and early intervention.

DIAGNOSIS

Having T2DM is associated with a much greater risk of NAFLD with approximately 70% of patients with T2DM having NAFLD when MRI-based techniques are used, as well as higher risk of having more advanced forms of the disease, such as fibrosis and cirrhosis (6,8,106). Despite all the current evidence, there is lack of awareness in primary care physicians and endocrinologists to evaluate patients with prediabetes or type 2 diabetes mellitus for NAFLD. Even if suspected to have NAFLD based on clinical characteristics, there is currently a lack of further investigations being undertaken as non-invasive biomarkers of the disease and even imaging, are not as reliable as wished and not available at every clinic. The widely accepted thought by primary care physicians, as well as many endocrinologists, is to not pursue any confirmatory testing to assess for the presence or degree of fibrosis, as it is believed to seldom change their management, except to re-emphasize lifestyle modifications and weight loss. However, few healthcare providers are aware about the efficacy of lifestyle changes and some currently available pharmacological agents to revert NASH, and even fibrosis, if done early and before the development of end-stage liver disease.

Early detection and treatment of NAFLD can lead to better histological and metabolic outcomes, including CVD, and improve overall morbidity and mortality. NAFLD is a diagnosis of exclusion, so it is imperative to eliminate all other causes of liver disease (such as, alcoholic liver disease, medication induced toxicity, viral or autoimmune hepatitis, hemochromatosis, alpha 1 antitrypsin deficiency, Wilson's disease, other) prior to the diagnosis of NAFLD. Often management may require referral to hepatology and developing multidisciplinary teams (107, 108). Once NAFLD

is diagnosed, there needs to be further testing to evaluate for the presence and severity of fibrosis (8).

Blood Tests

Plasma aminotransferases are considered an insensitive marker for the presence of NAFLD. It has been shown that the prevalence of NAFLD may be as high as 50% in patients with T2DM and "normal" (≤40 IU/L) plasma aminotransferases using ^{1}H-MRS for the detection of hepatic steatosis (109). Of note, 56% of these patients had a diagnosis of NASH on liver biopsy, highlighting that reliance on ALT/AST alone may be an inadequate approach for the systematic detection of NASH in endocrinology or primary care clinics (50). Maximos et al (110) have reported a comparable degree of NASH in patients with normal vs. abnormal levels of plasma aminotransferases, emphasizing the non-reliability of plasma aminotransferases as clinical biomarkers for presence or severity of disease, a finding consistent with the literature by others (6,9,111). Factors affecting elevation of plasma aminotransferases included adipose tissue insulin resistance and intra-hepatic triglyceride content, rather than hepatic insulin resistance (110). There is some evidence to suggest lowering the optimal threshold for considering plasma alanine transferase (ALT) as normal to be ≤30 U/L in men and ≤19 U/L in women (112). This increases the sensitivity of this screening method. Plasma ALT is usually more elevated than AST in the presence of NAFLD and NASH, unless there is advanced disease or cirrhosis, when AST usually increases.

Significant efforts have been made in finding the ideal biomarker panel for the diagnosis of NAFLD/NASH. Simple metabolic algorithms such as fatty liver index (using measures, such as BMI, waist circumference, triglyceride levels, and GGT) used for diagnosis of NAFLD have not been shown to be very reliable when compared with more accurate and advanced techniques, such as ^{1}H-MRS (113). It is not a test for the diagnosis of inflammation or fibrosis (114).

Several biomarker clinical scores (using different measures, such as AST, ALT, BMI, platelets, albumin, T2DM) have been developed to evaluate for the presence and degree of liver fibrosis (8). These tests are listed in the Table 3. Among these, only the NAFLD fibrosis score and FIB-4 have been confirmed across a broad spectrum of populations and considered the most reliable for the exclusion of advanced fibrosis (115). It is apparent that these scores are only able to distinguish relatively well between the two extremes – a population without evidence of NAFLD and a population with advanced fibrosis (F3-4). Most times, results fall in an intermediate or undetermined range, thus are not able to accurately classify patients in the spectrum of mild (F1) to moderate (F2) disease (9). These scores are also limited for use in population without T2DM. They have not been shown to be very reliable in this specific high-risk population of patients with T2DM (9).

Table 3. Biomarkers Available for use in Diagnosis of Advanced Fibrosis (Stages 3 or 4). Modified from reference (8)					
Test	**Parameters included**	**number**	**PPV**	**NPV**	**Patients unable to be classified "grey zone"**
NAFLD fibrosis score	Age, BMI, diabetes, AST/ALT ratio, platelets, albumin	733	82%	88%	24%
Fibrotest	Age, sex, total bilirubin, GGT, a2-macroglobulin, apolipoprotein A1, haptoglobin	267	60%	98%	32%
[†]FIB-4 index	Age, AST and ALT, platelets	541	80%	90%	30%
[†]BARD score	BMI, diabetes, AST/ALT ratio	827	43%	96%	N/A

NAFIC score	Ferritin, type IV collagen, insulin	619	36%	99%	15%
[†]Hepascore	Age, sex, total bilirubin, GGT, a2-macroglobulin, hyaluronic acid	242	57%	92%	11%

N/A, not applicable; NPV, negative predictive value; PPV, positive predictive value.

[†] No independent validation cohort included in the study.

Some commercially available tests based on a metabolomic profile have been tested as a novel means to evaluate for NAFLD or NASH and recently tested in patients with type 2 diabetes mellitus. These tests have shown some promise to distinguish between normal liver and NAFLD and also able to detect NASH in people without diabetes. However, when applied to a population with T2DM, these tests have not been as accurate as expected to predict presence of NAFLD, NASH or fibrosis (115, 116). There is an increasing interest in assessing the utility of novel biomarkers, such as plasma fragments of propeptide of type III procollagen (PROC3) for the detection of liver fibrosis in patients with T2DM. A recent study reported that PRO-C3 performed well (overall similarly to APRI or FIB-4) but with the added value of predicting histological changes in fibrosis stage with treatment (117). However, more studies are needed to determine its real value to monitor therapy. At the present time, available genetic tests include PNPLA3 and TM6SF2 and a few others (as described above), but they are not routinely performed at this time and limited to academic centers for research only. This is likely to change in the near future as more sophisticated genetic testing becomes available.

In summary, clinicians may use plasma aminotransferases or simple panels such as FIB-4 or NAFLD fibrosis score to identify patients at the highest risk of having NASH with advanced fibrosis (F3-4) in the clinic, but knowing that while the specificity may be acceptable ("rule out" advanced fibrosis or cirrhosis) the sensitivity is rather low. A screening strategy should include the above and imaging as described below as ultrasound and/or controlled attenuation parameter (CAP) have better sensitivity for the diagnosis of steatosis. The 2019 American Diabetes Association (ADA) guidelines for the first time recommend screening to identify liver fibrosis in patients with prediabetes or T2DM with elevated plasma aminotransferases and/or steatosis (118).

Imaging Modalities

MEASUREMENT OF INTRAHEPATIC TRIGLYCERIDES

Liver Ultrasound

Ultrasound is a relatively low-cost technique that is widely availability. Because of this it is routinely used for the diagnosis of NAFLD. However, it should be noted that the sensitivity of the test can be widely variable due to differences in operator technique and devices available, definition of steatosis, use of different echographic parameters to define steatosis, as well as the heterogeneity of the liver disease. While in one meta-analysis liver ultrasound was found to have a pooled sensitivity of 84.8% and specificity of 93.6% to detect hepatic steatosis of more than 20-30% (119), this literature is largely from liver clinics where disease severity is greater (i.e., more steatosis and better performance) but may not reflect the setting of primary care physicians or endocrinologists. More relevant was also the fact that the investigators calculated the sensitivity to diagnose moderate-to-severe fatty liver from the absence of steatosis, without considering mild-to-moderate NAFLD. However, clinicians are faced with many patients with NAFLD that have only mild-to-moderate intrahepatic triglycerides, emphasizing the importance of having simple imaging tools that can make the correct diagnosis in the clinic.

In a study by Bril et al (120), the authors compared in 146 patients the performance of ultrasound using a score from five echographic parameters for steatosis or liver fat quantified by ^{1}H-MRS. They used as the gold-standard histology (liver biopsy). They reported that the performance of liver ultrasound (parenchymal echo alone) was relatively poor but improved to an acceptable level when compared to ^{1}H-MRS when enhanced by the five echographic parameter score for steatosis was utilized. The greatest sensitivity of the ultrasound test was reached at a hepatic steatosis content of at least 12.5%. Below this threshold, the test was unreliable. Technological improvements may enhance in the near future the performance of liver ultrasound and its value in the management of patients with NAFLD.

Controlled Attenuation Parameter (CAP)

CAP is a relatively new imaging methodology to quantify steatosis. It is based on the principle that intrahepatic triglycerides delay ultrasound waves, so that when travelling through tissue with steatosis they will be attenuated when compared to normal liver tissue. The diagnostic range of CAP is from 100 to 400 dB/m. The higher the value the more suggestive of the presence of steatosis. The sensitivity of the test to diagnose hepatic steatosis was 68.8% and specificity was 82.2% in a meta-analysis of patients with biopsy-proven steatosis (121). Usually the cut-off of ≥280 dB/m is used to establish the diagnosis of steatosis. As discussed below, one advantage of CAP is that in addition to being a simple and useful point-of-care tool (often available in liver clinics), the estimation of CAP can be performed simultaneously with that of the liver stiffness measurement (LSM; Fibroscan®) and from the same liver region of interest, significantly facilitating clinical management although the test has its limitations when liver fat is only mildly elevated.

MR Spectroscopy

MR-based techniques have been the most accurate procedure to quantify liver triglyceride content (122). The use of ^{1}H-MRS has proven to be very accurate for quantification of intrahepatic triglyceride content, with the results correlating well with steatosis on histology (120). MR spectroscopy derived proton density fat fraction (MR-PDFF) has recently evolved into a simpler and easier to standardize method for multicenter studies examining the effect of liver steatosis of new agents for the treatment of NASH (9,108,114). It has shown better diagnostic and grading capabilities for liver steatosis when compared with controlled attenuation parameter modality using transient elastography (122). However, MR spectroscopy remains an expensive test available mostly in academic centers, and requires special expertise for performance and analysis of the test (108).

MEASUREMENT OF LIVER FIBROSIS

Liver Stiffness Measurement (LSM; Fibroscan®)

Liver fibrosis can be assessed in the clinic or bedside by measuring the "stiffness" of the liver. The LSM is estimated by using vibration controlled transient elastography or VCTE (Fibroscan®) to assess presence and severity of fibrosis. This modality also allows for a reasonably accurate quantification of the degree of fibrosis and hence, prognosis (108,92). It is a quick (10 minutes), easy, and economical tool for assessment, however the test requires a 3-hour fast, and in obese people liver fibrosis cannot be always estimated and performance is worse (particularly when BMI ≥40 kg/m^2) (108). At present, this test is not FDA-approved to be performed in patients with a pacemaker or during pregnancy.

MR Elastography

This modality is based on the same principle of liver "stiffness" as VCTE but it is a MR-based technique that has a sensitivity of 86% and specificity of 91% for assessment of degree of fibrosis (108). It is shown to be superior to VCTE, especially in diagnosis of early as well as advanced stages of fibrosis and cirrhosis. It is however much more expensive, requires special expertise to perform, and the current availability is limited. It also needs to take into account patient's size and weight, any metal implants, as well as anxiety and claustrophobia during the procedure (92,108).

Liver Biopsy

Liver biopsy remains the gold-standard for diagnosis of NASH and for assessing the degree/severity of fibrosis (94). It is the only modality to reliably distinguish between steatosis alone from NASH and advanced fibrosis and to eliminate other etiologies of liver disease 108,123-125).

The degree of liver disease on histopathology is graded on a score that has been developed, called the NAFLD activity score (NAS). NAS score ranges from 0-8 and includes three parameters that are graded separately – steatosis (0-3), hepatocellular ballooning (0-2), and lobular inflammation (0-3). The degree or stage of fibrosis is graded separately from 0-3. These scores and staging ranges allow for a more accurate and reproducible way of monitoring of disease (108,123,125). However, there are limitations involving liver biopsies as well due to the inter-pathologist variability in interpretation of grades and degree of steatosis, inflammation and fibrosis (92,124).

Despite all the current advances, there remains an urgent need for development of more cost-effective and reliable methods for non-invasive screening of NAFLD to ensure early and prompt diagnosis for the best treatment outcomes.

TREATMENT

The aim of treatment for patients with NASH is to delay or reverse the progression of fibrosis and improve NASH-related morbidity/mortality due to hepatic (cirrhosis and HCC) and extra-hepatic complications, mainly cardiovascular disease (CVD). Currently there is no pharmacotherapy approved by regulatory agencies for the treatment of NAFLD, although pioglitazone is recommended by the current guidelines as a choice for patients with or without T2DM, and vitamin E for patients without diabetes (92,124). The FDA has accepted 2 endpoints as valid ones for drug approval in clinical trials: a) Resolution of the histological findings that define NASH (necroinflammation) without worsening of fibrosis, and b) Reversal of ≥1 fibrosis stage without worsening of steatohepatitis/NASH (124,126,127). Despite the many ongoing efforts to find novel pharmacological agents the first-line of treatment will always be lifestyle modification including diet, exercise and weight loss (92,124), to combat insulin resistance and the related conditions like diabetes and obesity so closely related to NAFLD (128-130).

Weight loss: Lifestyle, Bariatric Surgery and Weight Loss Agents

Numerous studies have shown the beneficial effect of weight loss to improve hepatic steatosis. It has been reported that weight loss not only improves liver steatosis and other histological features of NASH (including fibrosis) but can decrease insulin resistance and blood pressure as well as improve atherogenic dyslipidemia (elevated LDL-C and triglycerides, low HDL-C) (92,131). In a meta-analysis of eight trials including 373 patients, improvement in hepatic steatosis was seen in patients who lost ≥5% of body weight, while NAFLD activity score (NAS) improvement was associated with weight loss of ≥7% body weight (132). In another randomized well-controlled trial paired with liver biopsy, weight loss and exercise program resulted in improvement of NASH. Moreover, this study showed that the magnitude of weight loss correlated strongly with improvement in histology (133). However, even with

intensive multidisciplinary lifestyle interventions, more than half of patients were unable to achieve the weight loss target (weight loss of ≥7% body weight) which makes patient compliance the main concern (132). Despite the presence of multiple studies that correlates weight loss with the improvement of histological disease in NASH, little is known about the long-term effect (i.e. beyond 1 year) of weight loss on liver histology (8).

Weight reduction of 10% by lifestyle modification may cause a significant regression of fibrosis (133,134). A greater and a more sustained over time decrease in weight loss with improvement in steatohepatitis, and even fibrosis, can be achieved by bariatric surgery (92,135,136). In a systematic review that included 21 observational studies of bariatric surgery in patients with NASH, an improvement in steatosis was reported in 18 studies, decreased inflammation was reported in 11 studies and improvement in fibrosis was reported in 6 studies (137). Only four studies reported some (minor) worsening of fibrosis (137). However, most bariatric surgery studies have some limitations: these include small size, lack of proper standardization of preoperative low-caloric diet, frequent dropouts, and often no standardized time after the repeat postoperative liver biopsy. Finally, there are no randomized clinical trials (RCT) that compare bariatric surgery versus conservative management in patients with NASH with liver histology as the primary endpoint (137,138). Weight loss agents had no specific liver benefit (131), but can help with weight control and cause improvement in plasma aminotransferases and liver histology (139,140).

Adding regular moderate-intensity aerobic exercise/ resistance training is highly encouraged as a lifestyle intervention for NAFLD. Exercise not only improves steatosis but the high cardio-metabolic risk profile, even in the absence of significant weight loss (92,124,141). In an uncontrolled study of 293 patients paired with liver biopsies, one year of structured exercise (walking 200 min/week) combined with a hypocaloric diet improved hepatic steatosis and necroinflammation (133). In order to sustain weight loss, most dietary recommendations for NAFLD reflect a combination of hypocaloric diet (500–1000 kcal/day energy deficit) with exercise (92,134).

Heavy alcohol consumption should be avoided by patients with NAFLD and NASH. Heavy drinking is defined as four standard drinks on any day or more than 14 drinks per week

in men, or more than three drinks on any day or seven drinks per week in women (92). There are no longitudinal studies reporting the effect of ongoing alcohol consumption on disease progression or the natural history of NAFLD or NASH.

Pharmacological Agents with Evidence from RCTs for the Treatment of NASH

Pharmacologic treatment has been extensively studied for patients with NASH with or without diabetes mellitus. For patients with NASH and T2DM, the typical initial therapy is with metformin. However, randomized controlled trials did not show improvement in liver histology (92,142).

Given that insulin resistance is a core feature in the pathogenesis of NAFLD/NASH, thiazolidinediones (TZDs), targeting the transcription factor PPAR gamma in adipose tissue and other tissues, has been tested in several RCTs in patients with NASH (3). Pioglitazone at the molecular level modulates glucose and lipid metabolism and improves adipose tissue and hepatic insulin signaling and insulin sensitivity, collectively leading to improved liver histology in patients with NASH (143-149). However, the exact mechanism of action in humans is unknown and likely involves other pathways, for instance, activation of a mitochondrial pyruvate carrier (MPC) and/or PPAR alpha effects that may enhance mitochondrial fatty acid oxidation. A recent study *in vitro* and *in vivo* suggested effects independent of activation of MPC (150). Of note, when rosiglitazone was compared to placebo in patients with NASH it did not show any improvement beyond a reduction in steatosis as hepatocyte necrosis, lobular inflammation and fibrosis were unchanged (151). This suggests that improvement in fibrosis is not necessarily due to PPAR gamma as rosiglitazone is strictly a PPAR gamma agonist while pioglitazone is a considered a weaker agonist that also has PPAR alpha activity. Of note, different PPAR gamma activators do not modulate function or increase the expression of identical genes. The expression profiles can vary, which can explain differential effects via PPAR gamma activation.

Pioglitazone has been the agent most studied to date in patients with and without diabetes and biopsy-proven NASH (143-149), as recently reviewed in-depth along with other medications to treat diabetes regarding their effect in NAFLD (152). Resolution of NASH with pioglitazone treatment has been fairly consistent across studies of 6 to 36 month duration and ranges from ~47% (or 29% placebo-subtracted) in patients without diabetes with pioglitazone 30 mg/day for 24 months (94), to ~60% (or ~40% placebo-subtracted) with pioglitazone 45 mg/day in those with prediabetes or T2DM treated for 6 to 36 months (143,148, 149). Taken together, these results suggest that pioglitazone might play a role in modifying disease progression and its natural history in patients with or without diabetes.

In addition, pioglitazone may improve the cardiometabolic profile of patients with NASH by reducing progression to diabetes and CVD. Many patients with obesity and NAFLD/NASH have (often undiagnosed) prediabetes. Pioglitazone has proven effective for the prevention of diabetes in subjects with prediabetes (153) and shown to ameliorate cardiovascular events in patients with metabolic syndrome or prediabetes with a history of a stroke. Recently, the IRIS study reported the effect of pioglitazone in patients that had taken ≥80% of the prescribed medication reduced stroke by 36%, acute coronary syndromes by 53%, and the combined endpoint of stroke/MI/hospitalization for heart failure by 39% (154).

However, it remains puzzling that for a population with such a high cardiovascular risk from having obesity, T2DM and NASH, the cardiometabolic benefits of pioglitazone are frequently dismissed because of potential side effects that can be mitigated with close monitoring: bone loss, weight gain (3-5%) (most usually associated with improved insulin action on adipose tissue, not edema), or lower extremity edema in ~5% but higher if on amlodipine or high-dose insulin (152,155). Consistent with diabetes prevention and CVD reduction (156-160), patients become more metabolically healthy despite weight gain (143,149). While pioglitazone improves left ventricular function in healthy patients with T2DM (161), it may trigger heart failure in patients who have fluid retention and subclinical (undiagnosed) heart failure with preserved left ejection fraction (HFpEF), also known as "diastolic dysfunction" (≤1%) (155). Obese patients with T2DM and NASH are more prone to HFpEF (162). Therefore, in our experience, this can be avoided if pioglitazone is not prescribed to poor candidates, such as those with long-standing history of severe CVD that could be associated with heart failure, baseline presence of unexplained shortness of breath or lower extremity edema, severe obesity (BMI ≥40 kg/m^2), or

longstanding diabetes on high-dose insulin. Concomitant use of amlodipine, that is often already associated with lower extremity edema, should also be avoided. The clinician suspecting HFpEF may consider ruling this condition out before initiating therapy. Options to this end are ordering a transthoracic echocardiogram or plasma N-terminal (NT)-pro hormone B-type natriuretic peptide (NT-proBNP), the non-active prohormone from BNP. Both BNP and NT-proBNP are released in response to changes in cardiac pressure with plasma levels increasing when heart failure develops or worsens (162).

There is significant controversy about the risk of bladder cancer with pioglitazone and unlikely ever to be resolved given the overall low frequency of bladder cancer in the general population. A recent 10-year prospective study was negative for bladder cancer (163) and there was no association found in a recent meta-analysis comparing patients who had been ever vs. never users of pioglitazone, but there was a small but significant association with 1–2 years (HR = 1·28 [1·08–1·55]) and >2 years (HR = 1·42 [1·14–1·77]) of exposure (164). In absolute terms, bladder cancer developed in <0.3% of patients both exposed and not exposed to pioglitazone. The numbers needed to treat for one additional case of bladder cancer ranged from 899 to 6380 (median of 2540), while the benefit for CVD and NASH ranged from 4–256 and 2–12, respectively.

Taken together, pioglitazone is an evidence-based treatment option for patients with and without diabetes and NASH (92). It is also a generic medication recommended by the current ADA and EASD guidelines as a low-cost option, along with sulfonylureas, for the management of T2DM. Pioglitazone is likely to become for patients with NASH what metformin is for the management of T2DM, an inexpensive and effective option offering liver histological and cardiometabolic benefit and likely to be combined with novel therapeutic agents under development.

Glucagon-like peptide 1 (GLP-1) receptor agonists are another group of pharmacologic agents widely used for the treatment of diabetes that also have significant cardiometabolic benefits. A recent review summarized the many studies that have tested GLP-1RAs in patients with NAFLD (152). Typically, treatment is associated with weight loss and a decrease in plasma aminotransferases and hepatic steatosis. In the only study to date examining their role in NASH, Armstrong et al (165) randomized 52 patients

with NASH to receive either liraglutide or placebo for 48 weeks. NASH resolved in nine patients (39%) who received liraglutide compared to two patients (9%) in the placebo group (RR 4.3; 95% CI 1.0-17). Patients who received liraglutide were less likely to have progression of fibrosis (9 versus 36 percent; RR 0.2; 95% CI 0.1-1.0). These results are consistent with most other controlled and uncontrolled trials with liraglutide and other GLP-1RAs that have consistently led to weight loss and a reduction hepatic steatosis on imaging and in plasma aminotransferases in patients with NAFLD (166). In contrast, DPP-IV agents have largely been ineffective in RCTs in NAFLD (166).

The sodium–glucose cotransporter 2 (SGLT2) inhibitors have a significant role in the management of patients with T2DM (167). They promote weight loss, reduce the risk of CKD and of heart failure, and decrease overall rates of cardiovascular events in patients with T2DM (168). Several studies in animal models of NAFLD have reported that this class of agents reverses hepatic steatosis and necroinflammation. Early studies reported improvements in plasma aminotransferases and hepatic steatosis (152). Recent controlled RCTs have reported a (modest) reduction in hepatic steatosis on imaging with canagliflozin (169) and dapagliflozin (170) in patients with T2DM and NAFLD. These findings combined with their attractive properties of weight loss and decreasing diabetic comorbidities would make them potentially valuable for combination therapy (i.e., pioglitazone) for patients with NAFLD, as shown from combination therapy trials in patients with T2DM (171-173).

Finally, it is important to mention vitamin E as it has been examined in RCTs for the treatment of NASH in patients with (149) and without (147) T2DM. In a study in patients with NASH but without diabetes, vitamin E showed improvement in the primary outcome, but had borderline efficacy for resolution of NASH (considered today a more relevant outcome) compared to placebo (36% vs. 21%; p = 0.05) and numerically appeared as less significant compared to pioglitazone (47%; p = 0.001 vs. placebo) (147). Recently, Bril et al (149) found that vitamin E alone appeared to not be as effective in patients with T2DM, as it failed to meet the primary outcome of a two-point reduction in the NAFLD activity score from two different parameters, without worsening of fibrosis. However, when vitamin E was combined with pioglitazone more patients on combination therapy achieved the primary outcome versus placebo (54% vs. 19%, P = 0.003) although the efficacy did

not seem to be greater than that with pioglitazone alone in previous trials (143, 148). Resolution of NASH occurred in both groups compared with placebo (combination group: 43% vs. 12%, P = 0.005; vitamin E alone: 33% vs. 12%, P = 0.04).

Other relevant group of agents tested in NASH include the lipid-lowering drugs (e.g. statins, colesevelam, omega 3 fatty acids, fibrates and niacin), which have not shown much success when studied in clinical trials in patients with NASH (174-179).

The Future: Many Agents on the Horizon for NASH

Given the rapid evolution of the field, with constant new drugs entering the arena of trials and others failing, we to refer the reader to recent in-dept reviews on the topic (180,181). Many pharmacological agents are being tested in phase 2 and phase 3 trials targeting a broad spectrum of pathways involved in the pathogenesis of NASH. Therapeutic targets of significant interest include farnesoid X receptor (FXRs), which regulate hepatic glucose and lipid metabolism (182). In the FLINT trial (183), in which obeticholic acid (manufactured by Intercept) was compared to placebo there was some evidence of histological improvement, including a mild effect on fibrosis that was recently confirmed in the Interim Analysis of the Phase 3 REGENERATE trial but showed no improvement in resolution of NASH (184). Unfortunately, a significant number of patients complain of pruritus and there was a worsening of dyslipidemia that can be mitigated by co-administration of statins (183). Several novel FXR compounds are in development (180,181).

As discussed, PPAR nuclear receptors play a key role in insulin sensitivity. In the light of their roles in NAFLD and NASH several combined PPAR agonists have been studied. Elafibranor (manufactured by Genfit), is a dual receptor PPAR-α/δ agonist that improves in insulin resistance and glucose/lipid metabolism (185). In the GOLDEN trial, a phase study 2b study, elafibranor 120 mg/day for a year led to a modest improvement in resolution of NASH compared to placebo in the subgroup with worse steatohepatitis (186). Another PPAR agonist is lanifibranor, a panPPAR agonist (PPAR-α/δ/ γ), is currently undergoing phase 2 clinical trials in NASH (187). Saroglitazar (by Zydus), is a dual PPAR-α/γ agonist with a predominant PPAR-α activity, reverses steatohepatitis in experimental

NASH models (188) and is undergoing clinical trials. A phase 2b RCT of MSDC-0602K by Cirius is expected to report results in late 2019 for the treatment of NASH. It is a compound designed to minimize PPAR gamma binding activity but to maintain binding affinity to a second cellular target of all TZDs that has been identified as the mitochondrial target of the TZDs (mTOT) or mitochondrial pyruvate carrier (MPC) (189). Other insulin-sensitizers in earlier stages of development include PXL-065 (by Poxel), an enantiomer of pioglitazone, and CHS-131 (by Coherus) a compound with PPARγ activity tested earlier in patients with T2DM.

Other pharmaceutical compounds being tested for the treatment of NASH aim at a variety of potential pathways. We will mention only a few examples for the reader to appreciate the broad spectrum of targets being studied. Aramchol (by Galmed) is a novel compound that downregulates stearoyl-CoA desaturase 1 (SCD1), a key enzyme involved in triglyceride biosynthesis (190). Inhibition of *de novo* lipogenesis (increased in NASH) by an inhibitor of acetyl-CoA carboxylase (ACC), the rate limiting enzyme in this pathway, is also being studied in RCTs in patients with NASH (GS-0976, Gilead) (191,192). Fibroblast growth factor (FGF)-19 functions as a hormone that regulates bile acid metabolism with effects on glucose and lipid metabolism (193). NGM282 (NGM Biopharmaceuticals) is an engineered analogue of FGF-19 for the treatment of NASH with promising early results (194). Several companies are testing analogues of FGF21 that have significant metabolic effects on glucose and lipid metabolism as well as hepatic fat (180, 181). Thyroid hormone receptor (THR) β-selective agonists, appear to specifically target the liver and improve steatohepatitis in animal models and early clinical trials in patients with NASH (195,196). Many other agents are being tested at this time.

CONCLUSION

Endocrinologists must be aware that NAFLD is a potentially severe disease in patients with T2DM, due to both its hepatic and extrahepatic complications. In 2019 the ADA included for the first time in its recommendations to implement regular screening for advanced fibrosis in all patients with prediabetes or T2DM with evidence of elevated plasma aminotransferases or steatosis, so an early diagnosis can prevent long-term complications (118). This is the first step of management while being aware of

the significant need for accurate and cost-effective diagnostic modalities and for continued research efforts for new treatments.

Figure 3 is a suggested algorithm to be used for endocrinologists and primary care settings when evaluating a patient with prediabetes or T2DM for the possibility of having NASH.

In the future, we anticipate that patients with T2DM will be routinely screened for NASH in the same way they are today for diabetic retinopathy or nephropathy.
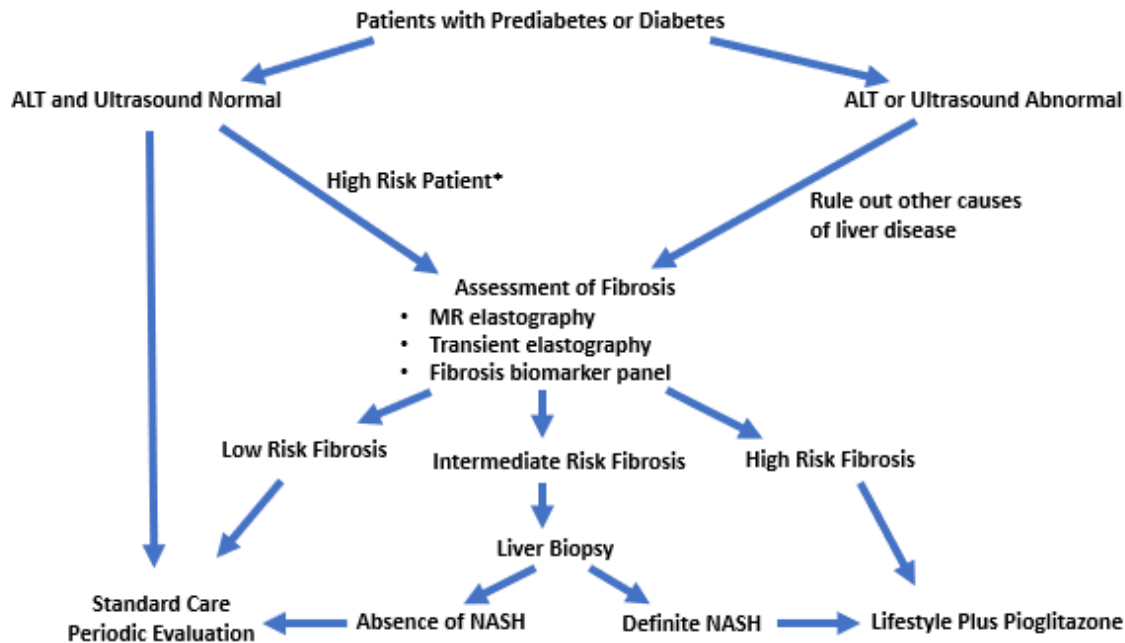
Figure 3. Management of patients with prediabetes or type 2 diabetes mellitus and suspected NAFLD. Based on figure from reference (8). *High risk patients include patients with type 2 diabetes > 10 years, A1c > 8.5%, triglycerides > 250mg/dl, evidence of steatosis based on MR imaging or controlled attenuation parameter (CAP), or genetic testing (PNPLA3 and/or TM6SF2).

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