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RISK FACTORS AND PROGNOSIS OF NON ALCOHOLIC STEATO HEPATITIS (NASH) TO ADVANCED SEVERE FIBROSIS: INTERVENTIONAL STRATERGY

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ABSTRACT

Non-alcoholic steatohepatitis (NASH) is a progressive form of Non-alcoholic fatty liver disease (NAFLD), characterized by steatosis, inflammation, and hepatocyte ballooning injury. The prevalence of NASH has been increasing with the trend of increasing risk factors. Despite the fact that the drug-based therapeutic interventions have not proved to be much effective, an appropriate intervention is needed to prevent disease progression in people with high risk-factors. Overall weight loss and exercise remain the key to improvement in the histopathological features of NASH including portal inflammation and fibrosis. Dietary supplementation of long-chain polyunsaturated fatty acids and antioxidant supplementation have been shown to be beneficial whereas high intake of fructose is implicated in NASH pathogenesis. Biomarkers including cytokeratin-18 (a marker of hepatocyte apoptosis and necrosis), markers of liver fibrosis, and markers of inflammatory stress and lipotoxicity may be beneficial in the differentiation of disease progression correlating with the gold standard. Additionally, understanding the role of genetic variations such as with PNPLA3, TM6SF2, and others, associated with NAFLD, in the metabolic and inflammatory pathway along with other risk factors may help in translating the information into clinical practice. In this review, we summarize current strategies for the treatment of NASH based on targeting the risk factors and controlling predisposing factors.

INTRODUCTION

NAFLD and NASH

Nonalcoholic fatty liver disease (NAFLD) is the most common hepatic alteration. The more severe form of NAFLD, nonalcoholic steatohepatitis (NASH), is characterized by inflammatory infiltration and hepatocellular damage, with or without fibrosis.

Prevalence of NAFLD and NASH

Nonalcoholic fatty liver disease (NAFLD) is an expanding health problem, which varies in prevalence among ethnic groups, occurring with an estimated global prevalence of 25% [1]. NAFLD associates with obesity, insulin resistance or type 2 diabetes and other metabolic abnormalities, such as dyslipidemia and hypertension, collectively termed metabolic syndrome. In high risk populations, the prevalence of NAFLD may be as high as 70%-90% [2,3].

Figure 1 Spectrum of NAFLD

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NAFLD covers a spectrum of pathological abnormalities. Although most patients have simple steatosis, around 7%-30% develop nonalcoholic steatohepatitis (NASH), that in at least a third of cases progresses to advanced fibrosis or cirrhosis.

Mechansim of NASH Progression

NASH is characterized by hepatocellular damage, inflammation and fibrosis [4,5]. In general, simple steatosis is considered a less severe form of NAFLD, although recent data indicate a possible risk of progression [6,7]. In contrast, NASH is a significant risk factor for the development of cirrhosis and hepatocellular carcinoma [8,9,10]. Although NASH was first documented more than 30 years ago [11], its pathogenesis is still not fully elucidated. Initially, a two-hit hypothesis, based on appearance of steatosis (first hit), followed by a second hit leading to inflammation, hepatocyte damage, and fibrosis, was proposed by Day and James [12]. While accumulation of triglycerides is necessary for the development of NASH, they may actually have a protective role against hepatocytes lipotoxicity, which is mainly induced by fatty acids and derived metabolites such as diacylglycerols, acylcarnitines or ceramides [13,14]. In addition, it is still unclear whether NASH develops sequentially, on the grounds of a fatty liver, or it is rather a de novo response to a lipotoxic environment. The multiparallel hypothesis proposed more recently [15] suggests that NASH is the result of numerous conditions acting in parallel, including genetic predisposition, abnormal lipid metabolism, oxidative stress, lipotoxicity, mitochondrial dysfunction, altered production of cytokines and adipokines, gut dysbiosis and endoplasmic reticulum stress. According to this hypothesis, hepatic inflammation in NASH may even precede steatosis. As more contributing factors are continuously identified, a more complex picture of NASH pathogenesis is emerging [16]

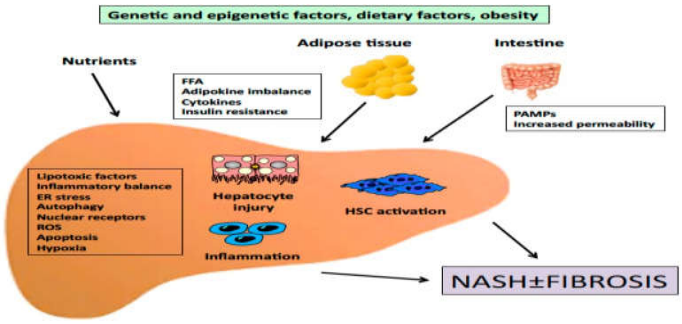

Figure 2 Outline of the pathogenesis of NASH. Signals generated inside the liver as a consequence of increased lipid accumulation, together with signals derived from extrahepatic organs cooperate to induce inflammation and fibrosis. FFA, free fatty acids; PAMPs, pathogen-associated molecular patterns; ER, endoplasmic reticulum; ROS, reactive oxygen species; HSC, hepatic stellate cell.

Interventional Stratergies

Genetic Factors: The relevance of genetic factor in the context of NASH has been recently and elegantly outline.[17]. A long list of genes potentially implicated in NAFLD appearance and progression has been reported, and these data have been the subject of a recent review [18].

A significant association with a single nucleotide polymorphism (SNP) was identified in patatin-like phospholipase domain-containing 3 (*PNPLA3*) on chromosome 22. The variant (rs738409 c.444 C>G, p.I148M), a non-synonymous cytosine to guanine mutation resulting in isoleucine to methionine conversion, correlates with increased hepatic lipid content and predisposes to fatty liver-associated liver disease, from simple steatosis to steatohepatitis, fibrosis and hepatocellular carcinoma [19,20]. *PNPLA3* encodes for a 481 amino acid protein, whose role has not been fully elucidated. It appears to function as acylglycerol hydrolase, acting on triacylglycerol, diacylglycerol, and monoacylglycerol [21,22]. Additional evidence indicates that PNPLA3 also acts as lysophosphatidic acid acetyltransferase [23,24]. Overexpression of the I148M variant in mouse liver promotes accumulation of triacylglycerol, increased synthesis of fatty acids and impaired hydrolysis of triacylglycerol [25]. Moreover, the *PNPLA3* genotype has been reported to influence liver storage of retinol and retinol serum levels in obese subjects [26], suggesting a potential role of PNPLA3 in regulating retinol metabolism and hepatic stellate cell (HSC) biology [27]. Remarkably, PNPLA3 has been recently shown to be expressed in hepatic stellate cells [28].

Carriage of a non-synonymous genetic variant in *TM6SF2* (rs58542926 c.449 C>T, p.E167K) on chromosome 19 (19p13.11) has been reported to correlate with steatosis and increased risk of advanced fibrosis in NAFLD patients [29,30], independently of other factors, including diabetes, obesity, or *PNPLA3*genotype. The minor allele frequency in one of the NAFLD populations tested was 0.12, compared to a frequency of 0.07 in a reference population. TM6SF2, is a transmembrane protein localized in endoplasmic reticulum (ER) and ER-Golgi compartments and functions as a lipid transporter [31]. The amino acid change E167K causes loss of function of TM6SF2 protein. Studies performed in cell lines showed that downregulation of *TM6SF2* reduces lipoproteins and apolipoprotein B (APOB) levels, and increases hepatic deposition of triglycerides and the amount and size of lipid droplets. In contrast, the size and number of lipid droplets diminishes when TM6SF2 is overexpressed, indicating that TM6SF2 plays a role in regulating hepatic lipid efflux [29,31].

A broad spectrum of other genes has been associated with NAFLD. Polymorphism was reported in genes involved in carbohydrate and lipid metabolism, insulin-induced pathways, as well as inflammatory response, oxidative stress and fibrogenesis. A study by Dongiovanni *et al*. reported that non-synonymous SNPs in ectoenzyme nucleotide pyrophosphate phosphodiesterase 1 (*ENPP1* or *PC1*) (rs1044498, K121Q) and insulin receptor substrate-1 (*IRS1*) (rs1801278, Q972R), are associated with insulin resistance, through impairment of insulin receptor-mediated pathways, such as reduced AKT activation, and promote fibrosis in NAFLD patients [32].

A functional non-synonymous variant (rs1260326, P446L) of glucokinase regulatory protein (GCKR) has also been associated with NAFLD [33]. This variant produces a GCKR with defective inhibitory function, leading to increased glucokinase activity and hepatic glucose uptake [34]. The resultant unimpeded hepatic glycolysis reduces glucose levels, inducing malonyl-CoA synthesis, a substrate for lipogenesis that causes liver fat deposition and impairs mitochondrial β-oxidation. A polymorphism in the solute carrier family 2 member 1 gene (*SLC2A1*), a glucose transporter, has been reported in NAFLD subjects. SLC2A1 downregulation in

hepatocytes results in lipid accumulation and oxidative stress [35].

Several genes involved in oxidative stress have been investigated. Two reports correlated the C282Y variant in hemochromatosis gene (HFE) with NASH and higher susceptibility to more severe disease, as fibrosis or cirrhosis [36,37]. However, these findings have not been confirmed by other studies [38,39,40]. Very recently, the rs641738 genotype at the *MBOAT7-TMC4* locus, encoding for the membrane bound O-acyltransferase domain-containing 7 was associated with more severe liver damage and increased risk of fibrosis in patients with NAFLD. This effect has been ascribed to changes in remodeling of the hepatic phosphatidylinositol acyl-chain

Dietary Factors

Lifestyle changes focusing on weight loss remain the keystone of NAFLD and NASH treatment [41]. Recent reports indicate that lifestyle modifications based on decreased energy intake and/or increased physical activity during 6-12 months cause improvement in biochemical and metabolic parameters and reduce steatosis and inflammation [42]. Conversely, increased consumption of sugar-sweetened food and beverages has been associated with NAFLD development and progression. High intake of fructose, used as food and drink sweetener, is implicated in NAFLD pathogenesis through several mechanisms. In addition, a fructose-enriched diet contributes to induce liver fibrosis in animal models of NASH [43]. Via the portal vein, dietary fructose reaches the liver in high concentrations, exerting a lipogenic action by activation of the transcription factors SREBP1 and ChREBP and subsequent induction of acetyl-CoA carboxylase (ACC) 1, fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1) [44]. These effects persist in liver-specific insulin receptor knockout mice, indicating that fructose stimulates lipogenesis independently of insulin signaling [45]. Fructose-induced de novo lipogenesis (DNL), enhancing malonyl-CoA concentration, inhibits mitochondrial β-oxidation and decreases mitochondrial ATP production [46]. In addition, fructose stimulates lipogenesis by inducing ER stress and subsequently activating the transcription factor X-box binding protein 1 (XBP1), which, in turn, upregulates lipogenic enzymes, as demonstrated in mice fed with a 60% fructose diet [47]. In concomitance, phosphorylation of fructose to fructose-1-phosphate leads to depletion of hepatic ATP and increase in ADP and inosine monophosphate (IMP), which is converted to uric acid [48], that promotes steatosis inducing mitochondrial oxidative stress [49]. Generation of reactive oxygen species (ROS) is also induced by fructose metabolism [50], and nutrient-derived ROS have been associated with enhanced steatosis via insulin-independent PI3K pathway [51]. Moreover, upregulating ketohexokinase, fructose potentiates its own metabolism and ketohexokinase inhibition leads to decreased fatty liver and reduced liver inflammation in high-fat/high-sucrose fed mice [52]. Finally, fructose-induced metabolic disorders can be mediated by epigenetic changes, such as alterations in genomic or mitochondrial DNA (mtDNA) methylation [53].

Emerging evidence underscores the role of cholesterol as a prominent risk factor for the pathogenesis of NAFLD/NASH. In humans a progressive increase in hepatic FC during NAFLD progression to NASH has been observed [54,55]. In experimental models increase in dietary cholesterol has been shown to promote hepatic inflammation and fibrosis [56,57,58], whereas a cholesterol-free diet ameliorates NASH [59]. The molecular mechanisms underlying FC accumulation during NASH development are multiple and only partially elucidated. Current data indicate that cholesterol homeostasis is dysregulated in NAFLD, due to an increase in cholesterol synthesis and uptake or dysfunction in cholesterol metabolism. Accordingly, the activity of two key regulators of cholesterol synthesis, HMGCR and SREBP2, is elevated in NASH patients. Similarly, expression analysis of genes involved in cholesterol metabolism reveals a number of altered pathways in individuals with NASH [60].

Main Features of Drug Induced NASH

Types of steatosis	Drugs involved in development of steatosis
• Macrovesicular steatosis	• Alcohol • Amiodarone • Chemotherapy (5-fluorouracil, tamoxifen, irinotecan, cisplatin, and asparaginase) • Glucocorticoids • Methotrexate • Total parenteral nutrition
• Steatohepatitis	• Amiodarone • Irinotecan • Methotrexate • Tamoxifen
• Microvesicular steatosis	• Aspirin (Reye syndrome) • Cocaine • Glucocorticoids • Nonsteroidal anti-inflammatory drugs (NSAIDS): ibuprofen and naproxen • Nucleoside reverse transcriptase inhibitors (NRTI) • Tetracycline (Intravenous administration of high doses) • Valproic acid

Biomarkers

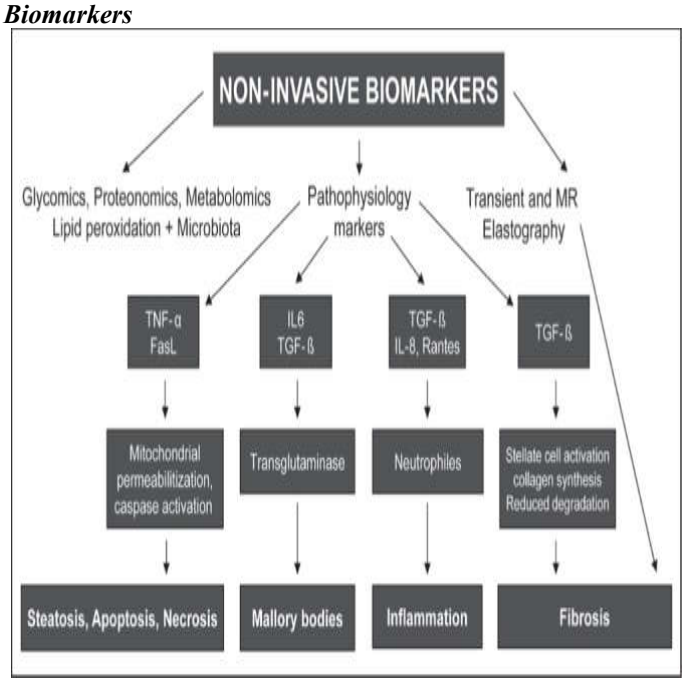

Figure 3 illustrates a possible strategy of identifying noninvasive biomarkers based on the methodology used and pathophysiology pathway[61].

Noninvasive biomarkers. Biomarkers can be measured using several technologies including glycomics, proteomics and/or

metabolomics, and imaging techniques such as magnetic resonance (MR) and elastography.

Noninvasive Markers to Differentiate Disease Progression Correlated With the Gold Standard

Correlation	Nonspecific, nonsensitive	Specific, sensitive
Slight correlation with severity of inflammation NAFLD/NASH; no correlation with fibrosis	Age, body mass index, insulin resistance, AST/ALT, platelet count, albumin, haptoglobin, α2-macroglobulin, bilirubin, γ-glutamyl transpeptidase	
Correlation with severity of metabolic syndrome NAFLD/NASH; no correlation with fibrosis or inflammation	Dyslipidemia: triglycerides, cholesterol, HDL, apolipoprotein A1, apolipoprotein B	
Oxidative stress correlated with inflammation steatosis and ballooning		Linoleic acid oxidation product: 13-hydroxy-octadecadienoic acid
Adipokine (adipocyte hormone), adiponectin, lower levels in NASH than in NAFLD with simple steatosis		Adiponectin
Adipokine, leptin, higher levels in obese NAFLD patients than in obese patients without steatosis		Leptin
Progression of inflammation		Ghrelin, ubiquitin sensitive markers for NASH
Sensitive inflammation markers		TNF-α, interleukins (IL-6; IL-8), RANTES and Fas ligand
Markers of liver fibrosis may help predict the evolutionary course of NAFLD		Hyaluronic acid, procollagen III N-terminal peptide, TGF-β and TIMP1
Cytokeratin-18 fragment: M30 – higher in NASH than in NAFLD with simple steatosis, correlation with inflammation, steatosis and fibrosis, no difference between healthy individuals and NAFLD patients; M65 – better predicating fibrosis F≥2		Markers of cell death CK-18 by apoptosis (fragment M30) and necrosis (M65)
Mitochondrial dysfunction cytokeratins: provide powerful predictions of risk in NASH[62]		CK-7/CK-18

- ALT Alanine aminotransferase;
- AST Aspartate aminotransferase;
- CK Cytokeratin;
- F1-F4 Fibrosis scores 1 to 4;
- HDL High-density lipoprotein;
- RANTES Regulated on activation, normal T cell expressed and secreted;
- TIMP Tissue inhibitor of metalloprotease;
- TGF-β Transforming growth factor-beta; T
- NF-α Tumour necrosis factor-alpha

Role of Hepatic Stellate Cells (HSCs) In Nash Progression

Liver fibrosis is a condition in which an excessive amount of extracellular matrix (ECM) proteins, like type I collagen, accumulates in the liver. This buildup of ECM occurs in most types of chronic liver diseases including NAFLD [63]. Although many cell types, including the hepatocytes and sinusoidal endothelial cells have been identified as contributors of ECM components, liver myofibroblasts, originally from HSCs (from the word of Latin origin, *stella*, meaning *star*), portal fibroblasts (PFs) or mesothelial cells are the major source of ECM [64]. The role HSCs play in fibrosis is unequivocal. Much data has demonstrated that HSC activation precedes fibrogenesis and that a lack of HSC activation halts the process [65-67]. Lipid accumulation, as that seen in NAFLD, triggers a profibrogenic response from HSCs; therefore an overview of fibrogenesis in NASH is critical to understanding NASH progression.

Although HSCs only make up about 1.4 percent of the liver cell population [68], their effect on overall liver homeostasis, particularly in cases of liver injury, is worthy of attention. HSCs are likely mesenchyme in origin, due to the fact that they produce alpha-smooth muscle actin (α-SMA) when activated and express vimentin and desmin [68]. HSCs reside in the space between hepatocytes and the liver sinusoidal endothelial cells, known as the space of Disse [69].

In healthy liver, HSCs exist in a quiescent state, storing vitamin A and lipids, a function, which led to an alternative name for HSC, the lipocyte [70, 71]. Upon liver injury, HSCs become highly proliferative, losing vitamin A and lipid droplets. In the same process, HSCs commence in mass production of a fibrotic extracellular matrix profuse with type I collagen [72] that allows the activated HSCs to be characterized as a myofibroblast-like cell.

HSCs, indeed, are the main cells involved in the production of extracellular matrix (ECM) in liver fibrosis [68, 73]. Other cell types like PFs and smooth muscle cells (SMCs) also contribute to the synthesis of connective tissue proteins as well [65]. For instance, the PFs, but not the HSCs of the hepatic sinusoid, play a predominant role in the early stage of cholestatic fibrosis when portal tracts are injured [65]. HSCs resemble and function in a similar manner as PFs when they are active. However, when quiescent, HSCs and PFs differ functionally as well as with respect to from which embryologic tissue they arise [70]. Different markers exist which can be used to distinguish between HSCs and PF. For example, recent research suggests that HSCs can be accurately distinguished from PFs based in expression of cytoglobin (CYGB): the CYGB protein is found in both quiescent and active HSCs but not in PFs after immunohistochemistry [68]. In addition, HSCs are positive for desmin and PFs are positive for elastin instead [67]

HSC activation involves two phases: the *initiation* phase and the *perpetuation* phase [73]. During the initiation phase, HSCs proliferate and become myofibroblast-like in response to proliferative and fibrogenic cytokines. Only activated HSCs express alpha2-macroglobulin, P100, CD95L, and reelin, which makes these proteins good identifiers for HSC activity [65]. There are many cells involved in activating HSC. For example,

hepatocytes, liver sinusoidal endothelial cells, macrophages, NK cells, and lymphocytes play roles in the activation process . Those cells secrete mediators that affect HSC activation. Of the mediators that are released, platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) are the two best-described growth factors. PDGF is involved in the signaling process required for HSC proliferation, while TGF-β promotes collagen production. The increase of ECM components (fibrillar collagens such as type I collagen) and inhibitors of matrix-degrading enzymes, like tissue inhibitor of matrix metalloproteinases (TIMP), occurs in the second phase of HSC activation-an event resulting in matrix accumulation, especially at sites where many activated HSCs reside[73] .

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