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Non-alcoholic Fatty Liver Disease: a sign of systemic disease

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HIGHLIGHTS

- NAFLD is the commonest form of liver disease in the United States and developed countries
- 20-30% of patients will progress to NASH, leading to fibrosis, cirrhosis, and HCC
- The liver has a central role in induction and progression of metabolic syndrome
- NAFLD is the hepatic component of the metabolic syndrome
- NAFLD is a systemic disease since different tissues and organs are involved

SUMMARY

Non-alcoholic fatty liver disease (NAFLD) is the most common form of liver disease and leading cause of cirrhosis in United States and developed countries. NAFLD is closely associated with obesity, insulin resistance and metabolic syndrome, significantly contributing to the exacerbation of the latter. Although NAFLD represents the hepatic component of metabolic syndrome, it can also be found in patients prior to their presentation with other manifestations of the syndrome. The pathogenesis of NAFLD is complex and closely intertwined with insulin resistance and obesity. Several mechanisms are undoubtedly involved in its pathogenesis and progression. In this review we bring together the current understanding of the pathogenesis that make NAFLD a systemic disease.

Keywords: fatty liver; NAFLD; NASH; insulin resistance; obesity

Abbreviations:

ACC: acetyl-CoA carboxylase

ApoB: apolipoprotein B

ApoCII: apolipoprotein C-II

APPL-1: adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and a leucine zipper motif

CD36: fatty acid translocase

CETP: cholesteryl ester transfer protein

ChREBP: carbohydrate-responsive element-binding protein

CKD: chronic kidney disease

COX: cyclooxygenase

CPT1: carnitine palmitoyltransferase 1

CVC: cardiovascular disease

DAG: diacylglycerol

DGAT2: diacylglycerol O-Acyltransferase 2

DNL: de novo lipogenesis

ECs: endocannabinoids

ER: endoplasmic reticulum

FA: fatty acid

FA: fatty acids

FABPpm: fatty acid binding protein

FASN: fatty acid synthase

FATP: fatty acid transport protein

FFA: free fatty acids

G6Pase: Glucose 6-phosphatase

GLUT: glucose transporter

HDL: high density lipoprotein

HL: hepatic lipase

HSC: hepatic stellate cells

HSL: hormone sensitive lipase

IL: interleukin

IRS: insulin receptor substrate

JNK: Jun N-terminal kinase

LDL: low density lipoprotein

LDLR: LDL receptor

LOX: lipoxygenase

LPL: lipoprotein lipase

MCP1: monocyte chemoattractant protein-1

MTP: microsomal triglyceride transfer protein

NAFLD: non-alcoholic fatty liver disease

NASH: non-alcoholic steatohepatitis

NF-κB: nuclear factor-κB

NO: nitric oxide

NOS: nitric oxide synthases

Nrf2: nuclear factor E2-related factor 2

PEPCK: phosphoenolpyruvate carboxykinase

PGE2: prostaglandin E2

PI3K: phosphoinositol 3-kinase

PKCε: Protein kinase Cε

PNPLA3: patatin-like phosholipase domain-containing 3

PPAR: proliferator-activated receptor

PUFA: polyunsaturated fatty acids

ROS: reactive oxygen species

SFA: Saturated fatty acid

SREBP: sterol regulatory element-binding protein

TG: triglycerides

TLR4: Toll-like receptor 4

TNFα: tumour necrosis factor alpha

VLDL: very low density lipoprotein

WAT: white adipose tissue

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) encompasses a broad spectrum of liver disorders characterised by fatty deposition in the liver in the absence of infection or significant alcohol intake. [1] It has varied histological spectrum, from fat accumulation within the hepatocytes to steatohepatitis, which may have associated fibrosis. [1] A significant proportion of people with NAFLD (20-30%) develop nonalcoholic steatohepatitis (NASH), which may lead to progressive liver fibrosis and cirrhosis, and hepatocellular carcinoma. [2] NASH carries 20% potential risk to evolve further into cirrhosis and thus it has become a leading cause of cryptogenic cirrhosis. [2]

The pathogenesis of NAFLD has not been fully elucidated. The liver has a central role in the regulation of lipogenesis, gluconeogenesis and cholesterol metabolism. [3] Hepatic lipid and glucose metabolisms are closely interrelated in the pathogenesis of liver disease and play a significant role in the induction of inflammatory, proliferative and apoptotic signal pathways within the liver. [3] The most widely accepted theory links metabolic syndrome, i.e. insulin resistance, to the development of hepatic steatosis and the progression to steatohepatitis. [4] Insulin resistance, obesity and fatty liver are arranged in kindred fashion and make an environment conducive to the development and progression of metabolic syndrome. NAFLD is common among obese and diabetic patients, but can also be found in the absence of these conditions. [5] NAFLD represents the hepatic component of metabolic syndrome. However, patients with metabolic syndrome are heterogeneous and have different expression of the disease. [6,7]

In this review we focus on the pathogenesis of NAFLD and its systemic correlation and aim to explore the possible future scenario to reduce the burden of the disease through the application of better understanding of the disease process.

1. CHANGES IN METABOLISM CONTRIBUTING TO NAFLD

Energy Intake and Diet Composition

Most lipids that accumulate within the liver derive from increased uptake of circulating fatty acids (FA) and increased endogenous synthesis of FA. Dietary intake affects the metabolism of the human body and plays a pre-eminent role in the development and progression of NAFLD. [8] Both energy intake and diet composition are indispensable in the genesis of the metabolic diseases. While saturated FA and trans-FA have adverse effects on lipid and glucose metabolism, monounsaturated FA and polyunsaturated FA (PUFA) seem to decrease insulin resistance, hepatic steatosis and inflammation in NAFLD patients. [9-11] Saturated FA (SFA), and trans-FA may promote inflammation due to lipotoxicity in the liver and alter the intestinal microbial flora leading endotoxemia. [12] SFA induces endoplasmic reticulum (ER) stress and leads to cellular dysfunction and apoptosis. [13] Conversely, PUFA positively modulate the expression of several genes involved in hepatic lipogenesis and oxidation of FA, such as sterol regulatory element-binding proteins (SREBP) and peroxisome proliferator-activated receptor (PPAR) alpha. [14,15] PUFA are precursors to eicosanoids that control inflammation and immunity. Among them, n-6 PUFA (also known as omega-6) are pro-inflammatory, whilst n-3 PUFA (omega-3) have anti-inflammatory properties. [16] Eicosanoids have important roles in the regulation of inflammation and are involved in several diseases, such as atherosclerosis, obesity, NAFLD and cancer. [17,18]

PUFA are metabolized by cyclooxygenase (COX) and lipoxygenase (LOX) into different types of eicosanoids. Proinflammatory eicosanoids include prostaglandin E2 (PGE2), which contributes to fat accumulation in the liver but can also induce COX2 enzyme leading to synthesis of interleukin (IL) 6; and leukotriene B4, which increases the production of reactive oxygen species (ROS) and inflammatory cytokines [19-21] COX2 induces several inflammatory mediators that are pro-fibrotic, being expressed in activate hepatic stellate cells, and contribute to apoptosis, necrosis, inflammation and fibrosis. [22,23] In many chronic inflammatory disease as well as in NAFLD, n-6:n-3 ratio is increased due to dietary imbalance. Studies have shown the potential role of n-3 PUFA in reversing many hepatic pathological changes induced by high-fat diet in obese mice and NAFLD patients. [24,25]

Dietary cholesterol also contributes to the development of hepatic steatosis and inflammation by alteration of mitochondrial function and modification of hepatic lipid composition. [26]

Alteration in cholesterol metabolism may contribute to disease severity and cardiovascular risk in NAFLD patients, since it can promote oxidative stress and progression to NASH. [27,28] However, several studies have shown that fruitarians and lean people can also develop NAFLD. It has been reported that even in the absence of obesity, fructose can cause severe liver damage, dyslipidemia, insulin resistance and progression to NASH. [29] Fructose may increase hepatic de novo lipogenesis (DNL) by upregulation of SREBP-1c and PPAR gamma, and may impair FA oxidation by downregulation of PPAR alpha, contributing to hepatic lipid accumulation. [29] Moreover, fructose can increase the transcription of phosphoenolpyruvate carboxykinase (PEPCK) and glucose transporter (GLUT) 2, increasing hepatic gluconeogenesis and contributing to hepatic insulin resistance. It can also induce the production of inflammatory mediators, oxidative stress, bacterial overgrowth and progression to NASH. [30,31]

Hepatic Lipid Metabolism in NAFLD: Triglyceride Accumulation

Triglyceride (TG) accumulation within hepatocytes is crucial in NAFLD. [32] FA that accumulate in the liver derive from peripheral lipolysis (free FA) from excessive food intake and from increased DNL, which is a complex pathway synthesing FA from glucose that can induce significant metabolic alterations if deranged. [32-35] In the liver, the rate of free FA uptake from plasma depends on the plasmatic concentration of FA and on the ability of hepatocytes to internalise FA. [34] Fatty acid transport proteins (FATP) and fatty acid translocase (CD36) are principally involved in FA uptake and are overexpressed in NAFLD (**Figure 1**). [33,34,36,37] As a consequence of excessive energy intake, FA esterification in the liver is enhanced with consequent increase in TG production via diacylglycerol O-acyltransferase 2, which is overexpressed in NAFLD. [38,39]

Several other alterations in hepatic lipid composition and FA metabolism have been demonstrated in NAFLD. [40,41] Increases in diacylglycerol (DAG), free cholesterol, n-6:n-3 FA ratio, decreases in phosphatidylcholine and arachidonic acid, and alteration of endogenous desaturase activities could all play a vital role in the progression to NASH. [41,42]

Glucose and fructose uptake are also enhanced in NAFLD. In the liver, GLUT2 regulates the influx of glucose in relation to the feeding state. Increased expression of GLUT2 has been noted with hyperglycemia, diabetes and NAFLD. [43,44] GLUT5, primarily a fructose carrier, is also expressed in the liver. [45] Although hepatic specific deregulation of GLUT5 in NAFLD

has not yet been investigated, GLUT5 may also be involved in the pathogenesis of NAFLD (**Figure 1**).

Role of White Adipose Tissue in the development of NAFLD

White adipose tissue (WAT) plays an active role in energy balance. It stores fat as TG during excessive food intake and releases free FA into the circulation when energy is needed. [46] FA in WAT derive from both plasma uptake and DNL. In normal conditions, insulin stimulates GLUT4-mediated glucose uptake in WAT and promotes re-esterification of free FA into TG for storage. [47] When dietary caloric intake is in excess, more FA are stored within the WAT. Lipoprotein lipase (LPL) activity, which contributes to lipids storage in the fed state, is increased resulting in a higher availability of free FA for WAT storage (**Figure 1**). [48,49] It has been demonstrated that in obesity LPL activity is increased in WAT. [50-52] However, LPL becomes resistant to insulin action in extremely obese subjects or after chronic hyperinsulinemia. [52]

Studies have reported the crucial role of transport proteins as CD36, FATP and fatty acid binding protein (FABPpm) that are overexpressed in obese patients. [53] While FATP and FABPpm are principally involved in FA transport, overexpression of CD36 in macrophages and adipocytes contributes to WAT inflammation and cell death (**Figure 1**). [54]

As caloric intake increases, expression of enzymes involved in TG synthesis is enhanced and causes further enlargement in adipocytes to store excessive TG. [55] With increase in adiposity, adipocytes become dysfunctional via the action of cytokines and insulin resistance. [55] Normally, insulin inhibits adipocyte lipolysis through suppression of the hormone-sensitive lipase (HSL). [56] During insulin resistance and in obesity, HSL is not inhibited resulting in enhanced lipolysis, reduced glucose uptake and impaired re-esterification of FA (**Figure 1**). [57,58] Impairment in adipocyte function and the production of inflammatory mediators, such as tumour necrosis factor alpha (TNFα), can reduce the expression of PPAR gamma, which is essential for adipogenesis and lipogenesis, and can impair TG storage in WAT. [55,59] Importantly PPAR gamma in WAT is also downregulated in the presence of insulin resistance and obesity.

Alteration in glucose metabolism in WAT is also important in insulin resistance, since GLUT4 expression is reduced in WAT in the presence of insulin resistance (**Figure 1**). [58] It has been reported that enhanced DNL in WAT is associated with improved glucose tolerance and insulin sensitivity in mice and humans. [58,60] In healthy individuals, DNL contributes

minimally to FA production in WAT. [61] SREBP-1c and carbohydrate-responsive element-binding protein (ChREBP) are both overexpressed in fatty liver, with SREBP-1c being a primary regulator of DNL in the liver but not in WAT. [58] Whereas knockdown of hepatic ChREBP in genetically obese ob/ob mice markedly improved the insulin resistance and liver steatosis, induction of ChREBP in WAT improved insulin sensitivity. [57] Since DNL in WAT may be important in glucose clearance and in regulating glycaemia, it could be possible that in WAT, ChREBP-induced DNL may have a positive effect on systemic insulin sensitivity. [57]

2. INSULIN RESISTANCE AND NAFLD

Insulin Action and Insulin Resistance in the Liver

NAFLD is frequently associated with features of insulin resistance. [62] For a long time, skeletal muscle has been considered the primary source of insulin resistance. [6] However, it has been proposed that skeletal muscle does not contribute to increased cardiovascular risk in the same way as the liver. [62] The liver is the central regulator of glucose and lipid metabolism and is particularly sensitive to insulin action. [6] Hepatic insulin resistance is associated with accumulation of TG and FA metabolites within the liver, such as fatty acyl-CoA, DAG, ceramides, and glycosphingolipid (**Figure 2**). [63] In normal conditions, insulin stimulates tyrosine kinase activity of the insulin receptor leading to phosphorylation of insulin receptor substrates (IRS) 1 and 2, with activation of downstream events that mediate the action of insulin. [64]

In fatty liver, DAG activates protein kinase Cε (PKCε), which subsequently inhibits the insulin receptor kinase. Phosphorylation of IRS1 and IRS2 is reduced, and their action is blocked. Activation of phosphoinositol 3-kinase (PI3K) and of protein kinase Akt2 is reduced, with the release of glucose via GLUT2 into the circulation (**Figure 2**). [63,65]

The exact mechanism that induces liver insulin resistance is still controversial. Instead of the DAG-PKCε–dependent mechanism, which may be common to all FA, it has been proposed that excessive intake of saturated fat may cause hepatic insulin resistance via activation of the toll-like receptor 4 (TLR4)/MyD88 pathway. TLR4 is a pro-inflammatory receptor and its activation by the adaptor protein MyD88 induces activation of IκB kinase, de novo synthesis of ceramides, ceramides accumulation and ceramides-mediated activation of protein phosphatase 2A, which directly inhibits insulin signaling at the level of Akt phosphorylation (**Figure 2**). [66,67]

Hepatic insulin resistance leads to increase in glucose production and hyperglicemia (**Figure 2**). [68] Furthermore, hepatic lipid and lipoprotein metabolism are also severely affected. [69] In NAFLD, insulin resistance contributed to increased hepatic FA uptake via action of insulin on CD36 and via increased lipolysis in WAT. [32] Hyperinsulinemia induces an increase in FA synthesis through DNL by expression of SREBP-1c in the liver. [3,33] Moreover, beta-oxidation is also impaired because of enhanced production of malonyl-coA via acetyl-CoA carboxylase 2 (ACC2), which is also induced by SREBP-1c. Malonyl-coA inhibits the enzyme carnitine palmitoyltransferase 1 (CPT1) which regulates beta-oxidation of FA in the mitochondria

(**Figure 2**). [33] However, mitochondrial beta-oxidation can be augmented in insulin resistance-associated NASH, as a compensatory mechanism to the increased uptake and synthesis of FA. [70] This involves activation of PPAR alpha and enhanced activity of CPT1, which can be stimulated by PPAR alpha and may lose affinity for malonyl-coA. [70-72] In any case, mitochondrial dysfunction, particularly deficiency in the respiratory chain, leads to overproduction of ROS, which damage several components of the cell with the genesis of oxidative stress and eventually apoptosis. [73] Furthermore, mitochondrial dysfunction can cause insulin resistance and induce overproduction of toxic lipid metabolites, which can further impair the action of insulin. [74] Oxidative stress together with other noxious stimuli can cause activation of the Jun N-terminal kinase (JNK), leading to IRS inactivation. In NASH, activation of JNK is responsible for insulin resistance, lipoapoptosis and fibrosis. [75-77] Apoptosis is a complex event and a key feature in NAFLD pathogenesis. Imbalance in apoptosis regulation is an important mechanism inducing progression of liver damage. [78] In NAFLD, several factors (i.e. SFA, PUFA) can induce apoptosis through different signalling networks including membrane death receptor-mediated cascade (extrinsic pathway), ROS formation, ER stress, lysosomal and mitochondrial dysfunction (intrinsic pathway). [13,22,23,75-77,79] In the liver, apoptosis promotes inflammation, fibrosis and cirrhosis, whilst in peripheral tissues (WAT, skeletal muscle, pancreas) apoptotic signals may contribute to the development of insulin resistance. [80-82 83 84]

Increase in hepatic fat also is associated with enhanced very low-density lipoprotein (VLDL) secretion from the liver in an attempt to maintain hepatic lipid homeostasis. [33] This mechanism is mediated by microsomal triglyceride transfer protein (MTP), a key enzyme for the assembly and secretion of VLDL that regulates the incorporation of TG into apolipoprotein B (ApoB). [85] In the circulation, TG-rich VLDL is converted to LDL (low density lipoprotein) by cholesteryl ester transfer protein (CETP). [86] In normal conditions, LDL are removed from the circulation by LDL receptors in the liver. [33] LDL receptor activity is reduced in NAFLD, while in NASH, MTP activity is reduced and VLDL secretion is impaired, with further retention of TG within the hepatocytes. [85,87]

Insulin Resistance and NAFLD

The liver is responsible for maintaining normal glucose levels during fasting. Hepatic accumulation of lipids reduces the hepatic responsiveness to insulin resulting in an increase of plasmatic levels of glucose and thus of insulin, producing a state of chronic

hyperinsulinemia. [88] Whether insulin resistance triggers lipid accumulation in the liver, or whether fat deposition in the liver alters the hepatic response to insulin is not clear. [6,88]

Hepatic FA can derive from diet, from peripheral lipolysis and from DNL. In patients with high-fat diet, diet itself could be responsible for accumulation of lipids in the liver and the development of NAFLD, as demonstrated by several animal models. [89] Moreover, dietary sugars enhance the endogenous synthesis of FA by inducing hepatic DNL. [90] Several other dietary components can differently contribute to the pathogenesis of NAFLD, as previously highlighted. [91] Nevertheless, it is also true that a high caloric intake results in obesity and insulin resistance, with direct stimulation of hepatic DNL through SREBP-1c and deregulated peripheral lipolysis with increased delivering of free FA to the liver, as demonstrated by the fact that NAFLD patients have increased levels of free FA. [92] In NAFLD patients, elevated serum levels of free FA are associated with features of metabolic syndrome, in particular obesity, hyperglycemia and hypertriglyceridemia, and correlates with inflammation and severity of liver damage. [93]

However, a group of patients with NAFLD are not obese. [94,95] Although visceral fat is also increased in lean patients with NAFLD, insulin resistance or alterations in adipokines secretion are not always found. In these subgroup of patients, dietary components (i.e. excessive intake of cholesterol and reduced intake of PUFA) may have a major influence and early development of hepatic insulin resistance and may be the key factor in the development of NAFLD and the subsequent metabolic alterations. [94,96,97] In this context, hyperinsulinemia is probably a consequence more than a cause of NAFLD. [88*59*]

It has been recently shown that lean patients with NAFLD have more severe liver inflammation and higher mortality rates than obese NAFLD patients. [98] It could be possible that obese and lean patients with NAFLD represent two distinct clinicopathogenic subgroups with genetic, environmental, and several other factors influencing pathogenesis and prognosis of the disease.

In obese and diabetic patients, peripheral insulin resistance may be initially responsible for the accumulation of fat in the liver, whilst diet can be the primum movens for hepatic steatosis and hepatic insulin resistance in non-obese patients.

3. OTHER FACTORS CONTRIBUTING TO NAFLD DEVELOPMENT AND PROGRESSION TO NASH

Adipokines, Inflammation and Progression to NASH

Adipose tissue remodeling is a continuous process that is pathologically accelerated in obesity. [99] Healthy WAT is composed of adipocytes and stromal cells, such as preadipocytes, endothelial cells, and immune cells that interact with adipocytes in the secretion of adipokines. [99-101] In normal conditions, expansion of WAT involves recruitment of adipogenic precursor cells, adequate angiogenic response and appropriate remodeling of the extracellular matrix, essential for the expandability and functional integrity of WAT. [100,102] In obesity, WAT expands massively, existing adipocytes enlarge, angiogenesis and normal remodeling of extracellular matrix does not occur in a sufficient way to ensure adequate blood perfusion with resulting hypoxia, which induces inflammatory and fibrotic changes in WAT. [102-105] Enlarged and inflamed WAT is characterized by macrophage infiltration. [102,105,106] Adipose macrophages produce several inflammatory cytokines contributing to the alteration in production of adipokines and to the pathogenesis of obesity-induced insulin resistance and NASH. [106] Adipokines are involved in body weight homeostasis, inflammation, coagulation, fibrinolysis, insulin resistance, diabetes, atherosclerosis, and cancer. [107,108] When visceral adiposity is increased, WAT produces more pro-inflammatory cytokines, such as TNFα, IL-6, and C reactive protein, whilst the production of adiponectin, a protective adipokine, is decreased (**Figure 3**). [109]

Adiponectin is mainly produced by adipocytes and has many important effects, including suppression of hepatic glucose production and hepatic lipogenesis, stimulation of glucose uptake by skeletal muscle, stimulation of FA oxidation in the liver and skeletal muscle, stimulation of insulin secretion and inhibition of pro-inflammatory cytokines (IL-6 and TNFα). [99] Adiponectin improves insulin resistance by actions on the liver and the skeletal muscle, as shown in obese animals. [110] Adiponectin binds to two specific receptors, AdipoR1 in skeletal muscle, and AdipoR2, mostly expressed in the liver. [111] In the liver, AdipoR2 interacts with APPL-1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain and a leucine zipper motif), which is involved in insulin signal pathways. APPL-1 activation triggers a cascade of events mediated by 5-AMP-activated protein kinase and PPAR alpha that leads to a change in the expression of several genes involved in glucose and lipid metabolism. [112] NAFLD patients have decreased serum

levels of adiponectin and reduced hepatic expression of adiponectin receptors, indicating a condition of adiponectin resistance. [111,113]

Adiponectin also has antifibrotic and anti-inflammatory effects, as studies have shown that patients with advanced fibrosis have low serum levels of adiponectin. [114,115]

In the liver, inflammatory cytokines are produced by Kupffer cells and hepatic stellate cells (HSC); these cytokines induce inflammation, cell death and fibrosis (**Figure 3**). [116] In healthy subjects, adiponectin stimulates the production of anti-inflammatory cytokines (IL-10), and reduces levels of pro-inflammatory cytokines (IL-6 and TNFα) by suppressing the activation of Kupffer cells and HSC, and may therefore ameliorate NASH and hepatic fibrosis. [117] The lack of adiponectin aggravates NASH, whilst adiponectin administration prevents progression to NASH in animal models. [118]

Another important protein in healthy WAT is leptin. Leptin is expressed mainly in the adipose tissue and interacts with different receptors in the central nervous system and peripheral tissues, including the liver. [99,118] Secretion of leptin is also proportional to body adiposity. Through hypothalamic pathways, leptin inhibits food intake and increases energy expenditure when energy is in excess. [99] Moreover, leptin suppresses hepatic glucose production and FA synthesis, whilst it stimulates FA oxidation in the liver and skeletal muscle, glucose uptake in skeletal muscle and insulin secretion. [99] Deficiency of leptin in animal models is associated with obesity, diabetes and NAFLD. [119-121] However, obese and NAFLD patients have increased levels of leptin, as a result of leptin resistance (**Figure 3**). [119,122]

The presence of steatohepatitis is the most important factor for progression to cirrhosis and end-stage liver disease in NAFLD. NASH is characterized by hepatic and systemic activation of immune and inflammatory response mediated by a cross talk between the liver, the gut and the adipose tissue. [123] The liver is able to produce a strong inflammatory response to several insults by activation of Kupffer cells, TLRs, lymphocyte, neutrophils and inflammasome. [124,125] Lipotoxicity is an important factor that leads to inflammation in the liver. However, not all the patients with NAFLD progress to NASH. It seems possible that inflammation originates outside the liver and that alteration in intestinal microbial flora, inflammation in WAT and circulating inflammatory cells play also an important role. [125] It is also evident now that a genetic predisposition to NAFLD, NASH and its complication exists. Patients with the patatin-like phosholipase domain-containing 3 (PNPLA3) gene variant I148M are at increased risk for NAFLD development and progression to NASH, have a greater amount of

fat deposition in the liver, and a more severe histology in terms of necro-inflammation and fibrosis. [126-128]

The pathogenesis of NASH is a complex process that starts with TG accumulation in the liver and progresses to inflammation and fibrosis via several mechanisms as described by the "multiple parallel hits" hypothesis. [129] In the first stages, hepatic TG accumulation represents a benign process as TG are considered to be an inert source of energy storage. With lipid overload, however, lipid metabolism is deranged and lipotoxicity is induced (**Figure 3**). [130]

A high caloric diet enriched in fat and fructose alters the microbial flora in the small intestine (microbiota), promoting intestinal inflammation and increasing gut permeability. [131] The gut and the liver are closely associated communicating via several mediators. [132] Integrity of the intestinal barrier is essential in the maintenance of a healthy gut-liver axis. [133] Patients with biopsy-proven NAFLD have increased intestinal permeability with disrupted intercellular tight junctions in comparison with health controls. [134] Commensal microflora normally provide a barrier effect in the gut and inhibit colonization by pathogenic bacteria. In patients with NAFLD and in several other diseases there is an imbalance between normal and pathogenic bacteria in the gut. [135] The human microbiota is a dynamic community and is susceptible to changes in environment and lifestyle. The composition of the gut microbiota is different in obese and lean individuals, and in patients with NASH and cirrhosis. [136] Patients with NASH have a lower percentage of Bacteroides in the stool and increased presence of Clostridium coccoides and alcohol-producing microbiota, such as Escherichia, with ethanol significantly contributing to gut permeability and to hepatotoxicity. [137-139]

The gut–liver axis plays a central role in the pathogenesis of obesity and NAFLD as intestinal microbiota interacts with the host immune system modulating intestinal permeability, inflammation, and insulin resistance. [131] Gut microbiota contributes to the pathophysiology of NAFLD in a number of different ways: by increasing body weight and FA synthesis, by contributing to insulin resistance, by alteration in the metabolism of choline with subsequent reduction in hepatic VLDL secretion, and by modification of bile acid metabolism. [132] Moreover, bacterial and endotoxin translocation due to increased gut permeability triggers the production of pro-inflammatory molecules (i.e. lipopolysaccharide) and cytokines that are hepatotoxic, these may be implicated in the development of insulin resistance and NASH. [133] Moreover, gut microbiota endotoxins, such as the lipopolysaccharide, interact with innate immune sensors, specifically with TLR4, mediating a state of systemic chronic, low-grade inflammation that affects the liver, WAT, the brain, islet cells and blood vessels, promoting NAFLD, insulin resistance, obesity, diabetes and

atherosclerosis. [140] The expression of TLR in macrophages induces the production of TNFα, IL-1b, chemokines and directly stimulates the release of pro-fibrogenic factors by hepatic stellate cells. [139]

In high-fat diet induced NAFLD, Kupffer cells are increased in number and activated by the lipopolysaccharide via TLR4, CD14, and lipopolysaccharide binding protein, leading to recruitment of leucocytes, activation of natural killer cells, production of pro-inflammatory cytokines (especially TNFα) and ROS, infiltration of monocytes within the liver, and alteration of the C-JNK and nuclear factor-κB (NF-κB) pathways, all promoting NASH progression. [141-146]

It has also been shown that trans-FA and fructose can directly induce hepatic steatosis and inflammation. [10,30,31,147] Fructose can directly alter the composition of gut microbiota, resulting in acquisition of a westernized microbiome with impaired metabolic capacity. [148] Finally, WAT inflammation and the alteration of secretion of adipokines also contribute to the development of NASH.

The Role of Oxidative Stress in NAFLD Progression

Oxidative stress is due to an imbalance between ROS and antioxidant systems. [149] Several pathophysiological events can trigger the production of free radicals in the liver, such as lipid peroxidation, hyperinsulinemia and hepatic iron overload. [150-152] In NAFLD, mitochondria are a major source of ROS. Mitochondrial dysfunction is crucial to the onset and progression of NASH. [153] Patients with NASH have swollen mitochondria with structural alterations, and impaired respiratory chain and beta-oxidation. [154]

Several chronic diseases like obesity, metabolic syndrome and fatty liver are associated with oxidative stress. In the liver, oxidative stress triggers an inflammatory cascade that produces progressive liver damage. [155] Patients with steatohepatitis have reduced glutathione level, decreased superoxide dismutase and catalase activity, and increased levels of hepatic cytochrome P450 2EI. [156]

ROS-mediated cell injury includes DNA damage, oxidation of FA in lipids and disruption of cell membrane integrity, oxidation of amino acids in proteins and protein instability, and release of pro-inflammatory cytokines. [157]

However, in liver the endoplasmic reticulum and peroxisomes have a greater capacity to produce ROS. [158] In NAFLD, there is an increased deposition of FA in the liver, while mitochondrial FA oxidation is altered with compensatory peroxisomal ß-oxidation and

microsomal ω-oxidation to reduce FA accumulation. However, enhanced oxidation in the peroxisomes and microsomes, as well as in the mitochondria, significantly contributes to the greater production of ROS in NAFLD. [159] Within the liver, lipid peroxidation results in production of aldehyde from PUFA. ROS and aldehyde can activate HSC leading to fibrosis and persistent chronic inflammation. [160] Kupffer cells are also an important source of ROS, nitric oxide (NO), cytokines and metalloproteinases that can activate the HSC inducing collagen synthesis and fibrogenesis. [161] Moreover, although still controversial, hepatic iron overload may also have a role in NASH development since patients with NASH have been shown to have higher level of iron. [162] In NASH, iron may contribute to oxidative stress, may induce necrosis of hepatocytes, inflammation through Kupffer cells, fibrosis due to activation of HSC and insulin resistance. [163,164] Moreover, iron depletion may improve liver damage and insulin resistance in NAFLD patients. [165,166]

In oxidative stress, together with the overproduction of ROS, there is a reduction in the antioxidant capacity of the cells, which is prevalently regulated by the transcription factor nuclear factor E2-related factor 2 (Nrf2). [167] Nrf2 activates the expression of several antioxidant response elements, including NAD(P)H:quinone oxidoreductase-1, glutathione transferase, and glutamate cysteine ligase. [168] In the livers of patients with NAFLD, Nrf2 is upregulated with subsequent increased expression of the antioxidant response elements, but their antioxidant function is impaired with disease progression to NASH. [169] In Nrf2-null mice fed with a methionine and choline-deficient diet, fat deposition was more severe, lower hepatic glutathione and enhanced lipid peroxidation were observed in comparison to mice expressing Nrf2 and especially to mice where Nrf2 expression was enhanced. [170] Moreover, steatohepatitis was more severe, its development was accelerated, suggesting that Nrf2 could represent a potential therapeutic target in NAFLD. [171,172]

Activated macrophages also generate excessive NO through the oxidation of L-arginine by the inducible form of nitric oxide synthases (NOS). NO is another potent free radical with strong cytotoxic and genotoxic effect through damage of proteins and DNA. [173] During oxidative stress, NO and superoxide can combine to form peroxynitrite, promoting nitration of tyrosine and damaging several cellular components. [174,175] Animals with fatty liver and obesity due to high fat diet have increased expression of inducible NOS with correlation to the presence of NASH and diabetes. [176] In NASH-related fibrosis, expressions of inducible NOS is increased, suggesting that NO can also have a role in inducing hepatic fibrosis. [177] Finally, patients with NAFLD and a polymorphism for the inducible form of NOS have increased risk for NAFLD and fibrosis. [178]

Insulin Resistance in Skeletal Muscle

Insulin resistance is defined as a reduced response of target tissues, such as the liver, skeletal muscle and adipose tissue, to the action of insulin. [179] Skeletal muscle is the predominant site of insulin-mediated glucose uptake in the postprandial state and is the major site for glycogen storage in humans. [180] Insulin resistance induces a reduction in glucose uptake and glycogen synthesis in the skeletal muscle. Dietary carbohydrates are thus diverted from the muscle to the liver and promote DNL, contributing to hyperlipidemia and NAFLD. [181,182]

Elevated levels of free FA in plasma are associated with skeletal muscle insulin resistance. [183] Several mechanisms have been implicated in the pathogenesis of free FA-induced insulin resistance. Free FA can directly stimulate the innate immune response via interaction with TLR4, which induces an inflammatory cascade via NF-κB, c-JNK and suppressors of cytokine signaling pathways, with consequent transcription of a variety of pro-inflammatory genes and production of pro-inflammatory cytokines. [182,184-186] An increase in skeletal muscle uptake of free FA and/or a reduction in FA oxidation due to mitochondrial dysfunction can also lead to intramyocellular accumulation of TG and lipid metabolites, such as long-chain fatty acyl-CoAs, DAG and ceramides. These metabolites can activate several kinases that interfere with the phosphorylation and activation of IRS, ultimately leading to a reduction in muscle glucose uptake and glycogen synthesis. [182,187] Furthermore, the change in the secretion of adipokines with overproduction of inflammatory factors such as TNFα, IL-6, monocyte chemoattractant protein-1 (MCP-1) and endocannabinoids (ECs), can disrupt skeletal muscle metabolism and contribute to insulin resistance (**Figure 3**). [186,188]

4. SYSTEMIC CORRELATION NETWORK OF NAFLD

NAFLD represents an independent risk factor for cardiovascular disease (CVD). [189] CVD is an important cause of death in patients with NAFLD. [190] NAFLD and CVD are strongly related since they have in common several metabolic derangements. [191] Moreover, NAFLD directly influences the pathogenesis of CVD by the release of pro-atherogenic factors, and atherosclerosis is common and more severe in patients with NAFLD, especially in its more advanced stages. [190,192,193] The pathogenesis of CVD in patients with NAFLD is still unclear. Atherogenic dyslipidemia, postprandial hyperlipidemia, endothelial dysfunction, hypercoagulability, cardiac dysfunction, inflammation, oxidative stress, low level of adiponectin and chronic kidney disease (CKD) can all contribute to the increased risk of CVD in patients with NAFLD. [189,192,194,195]

NAFLD is also associated with increased risk of CKD in patients with type 2 diabetes and also in nondiabetic and nonhypertensive patients, independently from the conventional risk factors for renal disease (i.e. duration and control of diabetes, cardiometabolic factors and medications). [196,197] As for CVD, NAFLD could be a marker of CKD. [198] However, NAFLD and especially NASH are characterized by a state of chronic inflammation and could instead represent an independent risk factor for CKD. [199-201]

Brain insulin resistance, neurodegeneration and cognitive impairment can all complicate obesity, type 2 diabetes and NASH, as demonstrated in experimental models of NAFLD. [202-204] Patients with NASH have increased rates of neuropsychiatric disorders and are at risk of cognitive impairment. [205,206] NAFLD, obesity and insulin resistance can induce a liver-brain axis of neurodegeneration mediated by toxic lipids, such as ceramides that can cross the blood-brain barrier due to their lipid-soluble nature, leading to progressive brain degeneration and cognitive impairment [202,203] [207] Additionally, hyperinsulinemia causes progressive injury to microvessels, producing a state of chronic cerebral hypoperfusion. [208] Moreover, gut microbiota also communicate with the brain via several different pathways and can influence brain function and behavior. [209]

Patients with fatty liver also have increased pancreatic fat content, a condition named as non-alcoholic fatty pancreatic disease. [83,210] It is not clear whether excessive pancreatic TG content (pancreatic steatosis) can cause beta-cell dysfunction. [211] Intrapancreatic fat and increased levels of free FAs can have a lipotoxic effect on islet beta-cells, causing impaired insulin production and beta-cells apoptosis. [83] [84] In a multi-ethnic sample of obese adults without diabetes, the correlation between pancreatic content of TGs and islet beta-cell

dysfunction was related to the different ethnicity of patients, suggesting a genetic predisposition for the development of pancreatic steatosis. [212] Free FA accumulate in several organs, including the pancreas, but in contrast with other organs, the contribution of FA in the pathogenesis of islet beta-cells dysfunction in humans is less clear. The increase in pancreatic lipid content may be a consequence and not the cause of impaired glucose metabolism in NAFLD and obese patients. [211]

CONCLUSION

NAFLD is a complex disease caused by different pathogenic processes as a result of the systemic interaction between the liver and several other organs. The liver has a central role in the pathogenesis of metabolic syndrome due to its central role in regulating lipid and glucose homeostasis. Dietary factors, gut microbiota, liver dysfunction and changes in adipose tissue, however, are all closely associated and inseparable in the pathogenesis of insulin resistance and NAFLD. To fully understand the pathogenesis of NAFLD and to develop new therapies it is essential to consider NAFLD as a multifactorial systemic disease involving the whole body.

The aim of this review was to help in understanding the pathogenesis of NAFLD and to try to cover most of the mechanisms involved. However, NAFLD is such a complex disease that it is almost impossible to cover in depth all mechanisms involved in one single review. Nevertheless, better understanding of a disease pathogenesis is essential to develop new and effective treatment strategies. Nowadays, many ongoing research efforts for NAFLD are directed towards the treatment of the clinical pathological changes of NASH to control disease progression and moderate the impact on patients' quality of life and social cost of NAFLD related complications. However, the natural history of any disease can be altered in several ways, including prevention. NAFLD is a complex systemic disease. Considerable progress has been made in the understanding of its pathogenesis. It is difficult to estimate the time that elapses between a scientific discovery and its application in the clinical field. However, the progress of science and the growth of knowledge are the only way to prevent disease onset, to achieve efficient diagnosis and to design new targets for drugs.

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IR: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript

JK: acquisition of data; analysis and interpretation of data; drafting of the manuscript

CA: acquisition of data; analysis and interpretation of data; drafting of the manuscript

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FIGURES

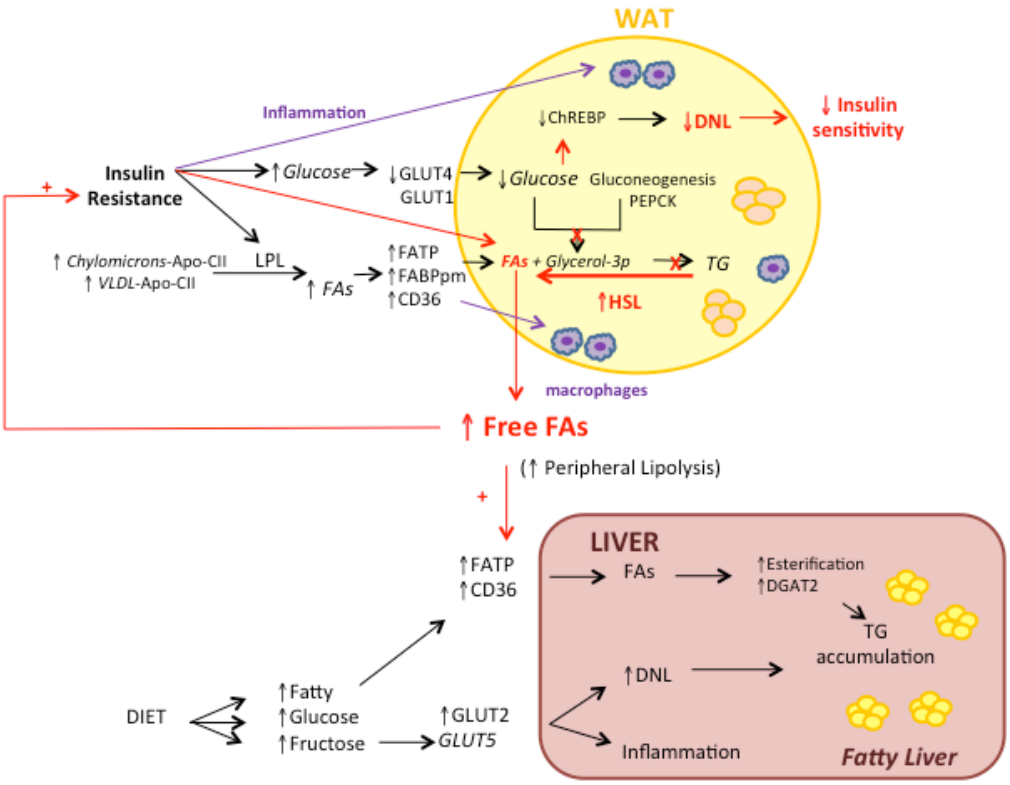


Figure 1: Changes in white adipose tissue metabolism and development of fatty liver

WAT stores FA in the form of TG. When dietary intake is in excess, more FA are delivered to WAT as chylomicrons and lipoproteins, which contain ApoCII. ApoCII is recognized by LPL, and this hydrolyzes TG back to FA. LPL activity is enhanced during calorie intake by several factors, including insulin. Membrane transporters take up FA, instead of being re-esterified as TG; lipolysis is increased due to insulin resistance and lipid overload. FA are then released into the circulation contributing to insulin resistance and fatty liver. Glucose uptake is reduced by downregulation of GLUT4. Glucose activates ChREBP. ChREBP enhances WAT DNL, which contributes to insulin sensitivity and improves glucose metabolism. Insulin resistance and CD36 overexpression induce macrophage accumulation and inflammation of WAT, which in turn releases several mediators that induce progression to NASH and worsen insulin resistance

Abbreviations: ApoCII: apolipoprotein C-II ChREBP: carbohydrate-responsive element-binding protein; DGAT2: diacylglycerol O-Acyltransferase 2; DNL: de novo lipogenesis;

FA: fatty acid; FABPpm: fatty acid binding protein; FATP: fatty acid transport protein; GLUT: glucose transporter; HSL: hormone sensitive lipase; LPL: lipoprotein lipase; NASH: non-alcoholic steatohepatitis; PEPCK: phosphoenolpyruvate carboxykinase; TG: triglyceride; WAT: white adipose tissue

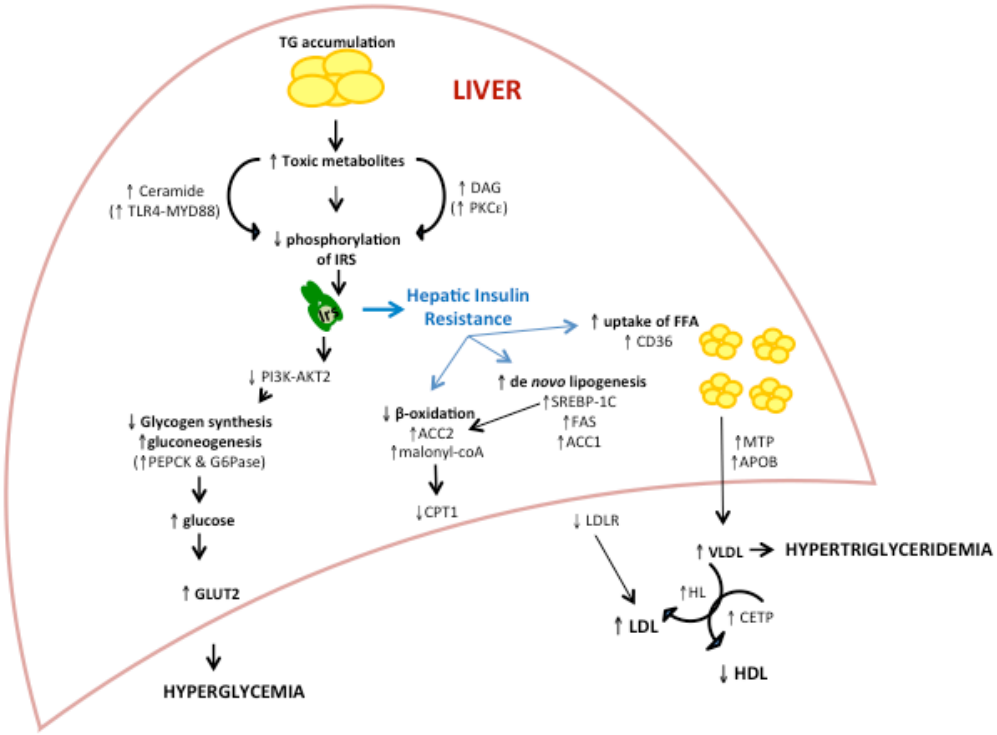


Figure 2: Mechanism of hepatic insulin resistance

As a consequence of TG accumulation and production of toxic metabolites, mitochondrial dysfunction and oxidative stress occur. Toxic metabolites (diacylglycerol and ceramides) are produced in the liver and interfere with the activation of insulin receptors (IRS1 and 2). The cascade of events that follows the activation of IRS is blocked. This activation is also impaired by the activation of JNK via oxidative stress. The liver becomes resistant to insulin action. Glycogen synthesis is reduced while gluconeogenesis is increased, and glucose is transported outside the liver via GLUT2, with subsequent hyperglycemia. Hepatic insulin resistance also alters the metabolism of lipids. Uptake of FA is increased, DNL is induced via insulin-activation of SREBP-1c and beta-oxidation is impaired, with induction of peroxisomal and microsomal oxidation. The liver tries to compensate the increase in TG by enhancing the export of TG as VLDL through the action of MTP. CETP and HL convert plasma VLDL into atherogenic LDL that are not cleared from blood due to a lower affinity for hepatic LDLR. This leads to hypertriglyceridemia, decreased HDL and increased LDL

Abbreviations: ACC: acetyl-CoA carboxylase; ApoB: apolipoprotein B; CETP: cholesteryl ester transfer protein; CPT1: carnitine palmitoyltransferase 1; DAG: diacylglycerol; FASN:

fatty acid synthase; FFA: free fatty acid; G6Pase: Glucose 6-phosphatase; GLUT2: glucose transporter; HL: hepatic lipase; IRS: insulin receptor substrate; JNK: Jun N-terminal kinase; LDLR: LDL receptor; MTP: microsomal triglyceride transfer protein; PEPCK: phosphoenolpyruvate carboxykinase; PI3K: phosphoinositol 3-kinase; PKCε: Protein kinase Cε; SREBP: sterol regulatory element-binding protein; TG: triglyceride; TLR4: Toll-like receptor 4

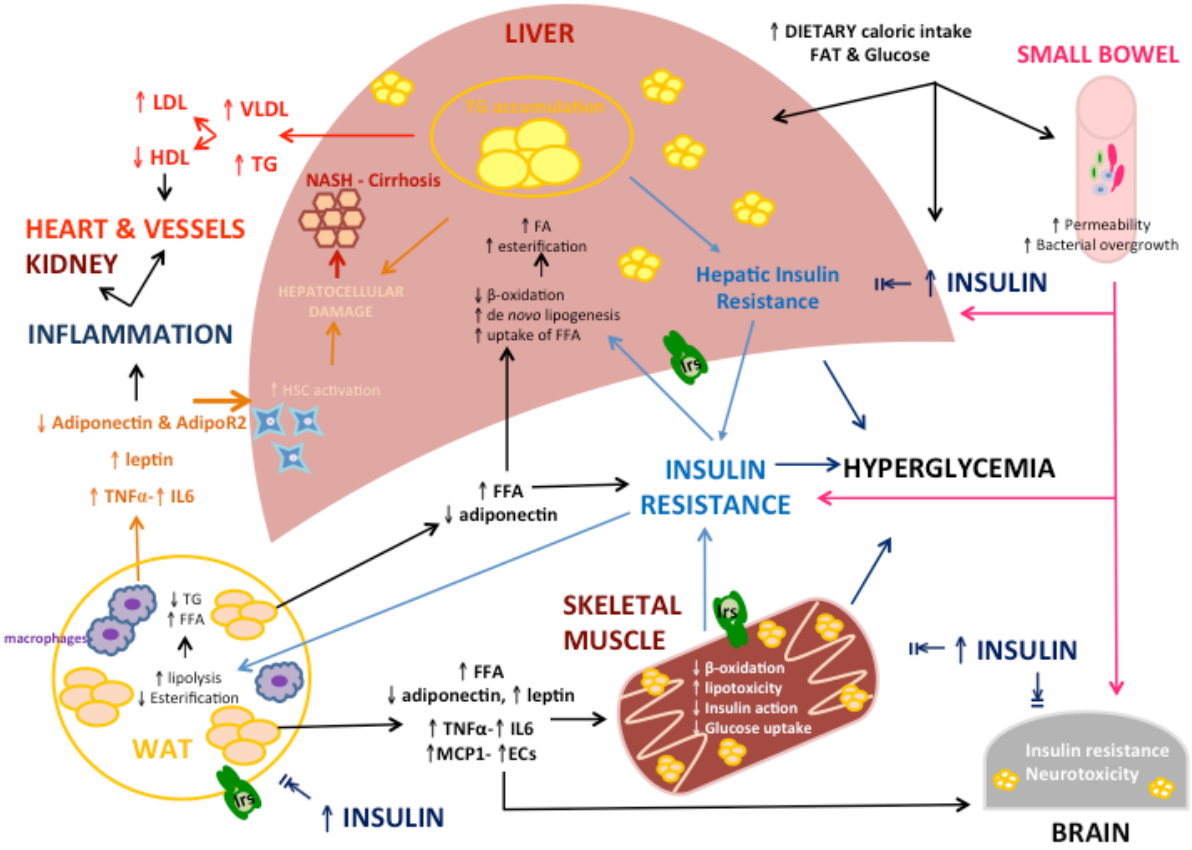


Figure 3: Interrelation between various organs in the pathogenesis of NAFLD and progression to NASH

Both development of NAFLD and progression to NASH result from the interaction between multiple organs and from different mechanisms of damage, indicating that NAFLD is a "systemic disease" that is caused by several mechanisms and that induces many dysfunctions in the whole body

Abbreviations: ECs: endocannabinoids; FA: fatty acid; FFA: free fatty acid; HDL: high density lipoprotein; HSC: hepatic stellate cells; IRS: insulin receptor substrate; LDL: low density lipoprotein; MCP1: monocyte chemoattractant protein-1; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; TG: triglycerides; VLDL: very low density lipoprotein; WAT: white adipose tissue

SUPPLEMENTAL TABLE: Summary of the genes and proteins described in the paper