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The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD)

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Abstract:

Nonalcoholic fatty liver disease (NAFLD) is increasingly prevalent and represents a growing challenge in terms of prevention and treatment. Despite its high prevalence, only a small minority of affected patients develops inflammation and subsequently fibrosis and chronic liver disease, while most of them only exhibit simple steatosis. In this context, the full understanding of the mechanisms underlying the development of NAFLD and non-alcoholic steatohepatitis (NASH) is of extreme importance; despite advances in this field, knowledge on the pathogenesis of NAFLD is still incomplete. The 'two-hit' hypothesis is now obsolete, as it is inadequate to explain the several molecular and metabolic changes that take place in NAFLD. The "multiple hit" hypothesis considers multiple insults acting together on genetically predisposed subjects to induce NAFLD and provides a more accurate explanation of NAFLD pathogenesis. Such hits include insulin resistance, hormones secreted from the adipose tissue, nutritional factors, gut microbiota and genetic and epigenetic factors. In this article, we review the factors that form this hypothesis.

Keywords: NASH, insulin resistance, lipotoxicity, gut microbiome, metabolic syndrome, PNPLA3

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome and is defined as the accumulation of fat in the liver in patients who do not consume excessive alcohol. The prevalence of NAFLD is 20-30% in adults and is higher in industrialized countries [1]. NAFLD is asymptomatic in most affected patients and is associated with obesity and features of the metabolic syndrome, namely hypertension, dyslipidemia, central adiposity and insulin resistance (IR) or diabetes [2, 3]. **The term NAFLD encompasses a wide spectrum of conditions, from simple accumulation of fat ('fatty liver' or steatosis) to steatohepatitis (NASH), fibrosis and cirrhosis with its clinical consequences** [4].

Despite its high prevalence, only a small proportion of subjects with NAFLD has NASH with consequent higher risk of liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC) (Figure 1). While patients with simple fatty liver have similar life expectancy to the general population, those with NASH have an impaired survival, due primarily to cardiovascular and liver-related causes. **This concept was recently challenged by two studies with longitudinal follow-up, which showed that advanced fibrosis, but not the presence of NASH (as diagnosed by the NAS score) predicted overall mortality in patients with NAFLD [5, 6].** The diagnosis of NAFLD remains one of exclusion and liver biopsy is still the gold standard to differentiate fatty liver from NASH and to stage fibrosis, although several noninvasive markers have been recently introduced for the latter [7].

The underlying mechanism for the development and progression of NAFLD is complex and multifactorial. Different theories have been formulated, leading initially to the 'two hits

hypothesis'. According to this, hepatic accumulation of lipids secondary to sedentary lifestyle, high fat diet, obesity and insulin resistance, acts as the first hit, sensitizing the liver to further insults acting as a 'second hit'. The 'second hit' activates inflammatory cascades and fibrogenesis [8]. This has been supported by animal models of obesity, such as the leptin deficient ob/ob mice, characterized by increased hepatic lipid accumulation, where a second insult is necessary to initiate inflammation and fibrosis [9].

However, it became rapidly evident that this view is too simplistic to recapitulate the complexity of the human NAFLD where multiple parallel factors, acting synergistically in genetically predisposed individuals, are implicated in the development and progression of disease.

Consequently, a multiple-hit hypothesis has now substituted the outdated two-hit hypothesis for the progression of NAFLD. In this review, we explore the potential mechanisms that are implicated in the pathogenesis and progression of NAFLD and that delineate the multiple-hit hypothesis.

1.1 Multiple hit hypothesis – an overview

Dietary habits, environmental and genetic factors can lead to the development of insulin resistance, obesity with adipocyte proliferation and changes in the intestinal microbiome.

Insulin resistance is one of the key factors in the development of steatosis/NASH and results in increased hepatic de novo lipogenesis (DNL) and impaired inhibition of adipose tissue lipolysis, with consequent increased flux of fatty acids to the liver [10]. Insulin resistance also promotes

adipose tissue dysfunction with consequent altered production and secretion of adipokines and inflammatory cytokines [11].

Fat accumulates in the liver in the form of triglycerides, and this happens contemporarily with increased lipotoxicity from high levels of free fatty acids, free cholesterol and other lipid metabolites: as a consequence, mitochondrial dysfunction with oxidative stress and production of reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress associated mechanisms, are activated [12].

Also, altered gut flora leads to further production of fatty acids in the bowel, increased small bowel permeability and thus increased fatty acid absorption and raised circulating levels of molecules which contribute to the activation of inflammatory pathways and release of proinflammatory cytokines such as IL-6 and TNF- α [13].

In subjects predisposed by genetic factors or epigenetic modifications, all these factors affect hepatocyte fat content and liver inflammatory environment, thus leading to a state of chronic hepatic inflammation (Figure 2) through heterogeneous hepatocellular damage pathways, with possible progression to hepatocellular death (for both direct toxicity and apoptosis activating mechanisms), activation of hepatic stellate cells and deposition of fibrous matrix.

Although the dictum was that steatosis always precedes inflammation, it is now recognized that NASH can be the initial liver lesion: the timing and combination of genetic, external and intracellular events rather than the simple sum of hepatic insults result in different pathways which lead to steatosis or NASH respectively [14].

This could be due to shortcomings of the NAS score, as the presence and grading of steatosis has a disproportionate impact compared to lobular inflammation and ballooning, while portal inflammation is not part of the score [15]. Alternatively, lobular inflammation and ballooning could be epiphenomena similarly to simple steatosis, not related to the activation of fibrogenic pathways.

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2. Fat metabolism, lipotoxicity and insulin resistance

Fat accumulates in the liver of patients with NAFLD mainly in the form of triglycerides [16, 17]. Triglycerides derive from esterification of glycerol and free fatty acids (FFA). Once synthesized, triglycerides enter storage or secretory pools, which have distinct rates of turnover. FFAs derive either from diet or from the adipose tissue via lipolysis and/or from hepatic DNL. Once in the hepatocytes, FFA undergo acyl-CoA synthases activity and form fatty acyl-CoAs, which may enter either esterification or β-oxidation pathways [18].

Triglyceride accumulation is not hepatotoxic per se and could represent a defensive mechanism to balance FFAs excess as demonstrated in mice models [19, 20]. The inhibition of triglyceride incorporation into new VLDL by blocking MTP causes impaired triglyceride secretion and therefore triglyceride accumulation without liver injury [21]; inhibition of DGAT2 expression, a key enzyme involved in triglyceride formation, resulted in a reduction of intrahepatic triglycerides and subsequent increased FFA oxidation and worsening of steatohepatitis in mice models [20]. Therefore increased triglyceride concentration is an epiphenomenon which happens simultaneously with toxic metabolites generation, lipotoxicity and liver injury [22].

Hepatic DNL can be increased by activation of transcription factors such as sterol regulatory element-binding protein-1 (SREBP-1), carbohydrate response element-binding protein (ChREBP) and peroxisome proliferator-activated receptor (PPAR)-γ [23]. SREBP-1 is a transcriptional factor present as three isoforms: while SREBP-1c regulates the activation of DNL and is stimulated by insulin [24], SREBP-2 is involved in cellular cholesterol homeostasis, therefore their dysregulation is involved in hepatic fat accumulation [17]. ChREBP is activated by glucose and increases DNL but also provides more substrate for triglyceride and FFA synthesis. Irrespective of the underlying mechanism, patients with NAFLD have increased DNL compared to controls, which is not suppressed on fasting, and higher nocturnal plasma levels of FFAs [25].

Among the insulin receptors, insulin receptors substrate 2 (IRS-2) can work, when activated, as a regulator of SREBP-1c, influencing DNL [26]. In states of insulin resistance IRS-2 is down-regulated, therefore SREBP-1c is over-expressed and DNL is up-regulated [27]. Also, β-oxidation of FFAs is inhibited in insulin resistance states, thus further promoting hepatic lipids accumulation [28].

FFAs in hepatocytes may induce defects in insulin signaling pathways through serine-kinase activation and contribute to insulin resistance state [29] [30]. Furthermore, insulin has a potent action to suppress adipose tissue lipolysis: in insulin resistance states, this suppression is impaired, resulting in increased efflux of FFAs to the liver [31]. Other pathways of fat disposal such as impaired hepatic fatty acid oxidation or reduced synthesis or secretion of VLDL have less importance in determining fat accumulation and lipotoxicity in NAFLD [32]. Autophagy,

which regulates lipid metabolism, decreases in liver steatosis, thus resulting in a vicious cycle of lipid accumulation and further suppression of the autophagic function [33].

Insulin resistance is a cardinal feature of NAFLD and is more prevalent in NASH compared to simple steatosis [34]. Patients with hepatic steatosis and NASH but without type 2 diabetes have decreased insulin sensitivity [35, 36]. Insulin resistance is one of the 'multiple hits' predisposing to the development of NAFLD and progression to NASH, critical for the establishment of lipotoxicity, oxidative stress and inflammatory cascade activation [8]. In patients with NAFLD, both genetic and environmental factors further interfere with the insulin signaling cascade and therefore contribute to the maintenance and worsening of insulin resistance: serine phosphorylation of insulin receptor substrate by inflammatory signal transducers such as c-jun N-terminal protein kinase 1 or inhibitor of nuclear factor-jB kinase-b (IKKb) [37], activation of nuclear factor kappa B (NF-kB) and SOCS (suppressors of cytokine signaling) [38] are some of the mechanisms that may disrupt insulin signaling in patients with NAFLD.

2.1. Mitochondrial dysfunction

Structural and functional alterations in mitochondria contribute to the pathogenesis of NAFLD. Structural alterations encompass depletion of mitochondrial DNA, morphological and ultrastructural changes, while functional alterations include the respiratory chain and mitochondrial β-oxidation [39].

If mitochondrial or peroxisomal function cannot handle the increased lipid flux, respiratory oxidation may collapse with impairment of fat homeostasis, generation of lipid-derived toxic

metabolites and overproduction of ROS [40]. All these molecules activate inflammatory pathways contributing to hepatocytes necroinflammation [12] and worsening of mitochondrial damage.

It has been indeed demonstrated that there is a correlation between insulin resistance, obesity, TNF-α levels and mitochondrial dysfunction [41]. Furthermore ROS, together with oxidized LDL particles, may activate Kupffer and hepatic stellate cells, leading to inflammation and fibrosis [42].

Whether mitochondrial dysfunction is a key pathogenic event in NAFLD or the consequence of an altered lipid metabolism still remains unclear.

2.2. Endoplasmic reticulum stress

An increased protein synthetic input, a primary dysfunction of the ER or lack of ATP can lead to unfolded proteins accumulation within the ER, activating the so called 'unfolded protein response' (UPR), which is an adaptive response aiming to resolve ER stress. UPR activation involves adaptive mechanisms such as reduction of protein synthesis, increased capacity for protein transit through the ER, increased protein folding and transport and activation of pathways for protein degradation, which help in resolving the protein-folding defect which would otherwise lead to initiation of apoptosis [43].

In NAFLD, factors that induce UPR include hyperglycemia, mitochondrial injury that depletes ATP, hypercholesterolemia, depletion of phosphatidylcholine and oxidative stress [44].

UPR leads to the activation of c-jun terminal kinase (JNK), an activator of inflammation and apoptosis: its silencing in animal models leads to decreased steatosis and steatohepatitis [45], while its activity has been showed as one of the features which differentiate subjects with NASH from those with simple steatosis [46]. Furthermore, JNK activity is linked to impairment of insulin signaling and subsequent development of diabetes mellitus [47].

Another consequence of UPR is the activation of SREBP-1c pathways and therefore maintenance of liver fat accumulation and further aggravation of ER stress and of UPR [48].

X-Box binding protein-1 (XBP-1) is one of the main regulators of UPR and interacts with the PI3K insulin signaling pathway, with increased nuclear translocation induced by insulin [49]. The interaction of PI3K and XBP-1 is both modulated by and modulates the cellular response to ER stress [50]. XBP-1 has been therefore proposed as the missing link between steatosis, insulin resistance and inflammation [51].

2.3. Inflammatory state in NAFLD: IL-6 and TNF- α

Increased levels of FFAs and consequent lipotoxicity, insulin resistance, peripheral adipose tissue dysfunction and gut derived endotoxins concur in activating and maintaining the production and release of pro-inflammatory cytokines, both systemically and locally in the liver.

Two main inflammatory pathways, JNK-AP-1 and IKK-NF-κB, are critically involved in the development of the chronic inflammatory state in NAFLD [52]. As previously mentioned, JNK is a member of mitogen activated protein kinases, associated with activation of apoptosis and development of NASH.

Nuclear factor-jB kinase-b (NF-kB) is a transcription factor and a primary regulator of inflammatory activation, and its IKK2 subunit is the major component required for its activation during the acute inflammatory response [53]. Persistent NF-κB pathway activation has been shown in animal models of NAFLD [54] as well as in patients with NASH [55]. IKK2 over-expression and persistent activation of NF-kB in hepatocytes lead to a chronic inflammatory state and insulin resistance [54].

The key role of the hepatic production of cytokines in the progression from steatosis to NASH is supported by studies in animal models demonstrating that hepatic exposure to increased levels of proinflammatory cytokines leads to histological changes typically seen in NASH, such as hepatocyte necrosis and apoptosis, neutrophil chemotaxis, activation of hepatic stellate cells and production of Mallory bodies [56]. Furthermore, serum and hepatic levels of TNF-α are increased in patients with NASH and correlate with histological severity of liver damage [57].

In addition, inflammation and NF-kB activation can promote carcinogenesis, and therefore the chronic inflammatory state of NAFLD may play a role in HCC development [58].

2.4. Inflammasome activation

Inflammasomes are cytoplasmic multi-protein complexes, consisting of caspases and molecules derived from pathogen microorganisms or liberated by injured cells, which can trigger inflammation. They act as sensors of endogenous or exogenous pathogen-associated molecular pattern molecules (PAMPs) or damage-associated molecular patterns (DAMPs). As part of the innate immune system activity, inflammasomes contribute to immune activation in response to different stimuli [59]. They regulate effector pro-inflammatory cytokines by activation of

families of germ-line encoded pattern recognition receptors (PRRs) as the Toll-like receptors (TLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs). Release of DAMPs can be induced by several mechanisms such as cell necrosis, apoptotic cells that are not removed by phagocytes and oxidative stress [8].

The activation of inflammasomes in response to FFAs, oxidative stress and other pro-inflammatory metabolites seen in NAFLD with consequent production of IL-1β could have an important role in the development of NASH, by inducing suppression of peroxisome proliferator-activated receptor-α (PPAR-α), and promoting the indirect effect of TNF-α-induced cell death [60]. Inflammasomes could also have a direct effect on hepatic stellate cells and promote fibrosis [61]. In NASH, saturated fatty acids up-regulate the inflammasome, trigger the release of danger signals from hepatocytes in a caspase-dependent manner and induce sensitization to LPS for IL-1β release in hepatocytes [62]. Conversely, mice with NLRP3 inflammasome loss of function are protected from diet-induced NASH [63].

Kupffer cells are important for inflammasome activity [64]: they express high levels of IL-1β mRNA and their reduction results in decreased total liver IL-1β mRNA and also decreased serum levels of IL-1β [65]. In murine models of NASH, signaling through TLR9 activation stimulates Kupffer cells to produce IL-1β with consequent steatosis, inflammation and fibrosis [60, 65]. Modulation of the Kupffer cells phenotype may represent a possible therapeutic antifibrotic strategy [66].

Accordingly, expression of inflammasome-associated proteins (NLRP3, pro-IL-1β, and pro-IL-18) is significantly higher in patients with NASH compared to those with simple steatosis [63].

3. Adipose tissue dysfunction

Adipose tissue is not inert as traditionally thought but has an endocrine function and secretes hormones (adipokines) such as leptin and adiponectin [67].

Obesity-related adipocyte hypertrophy and/or insulin resistance result in an imbalance of adipokines that may profoundly affect not only the adipose tissue itself but also the liver [68].

Adipose tissue contributes to the maintenance of low-grade inflammatory states by producing pro-inflammatory cytokines: serum levels of IL-6 and adipocytes expression of TNF-α are increased in obese patients and subsequently decline following weight loss [69, 70]. Furthermore, increased expression of inflammatory genes and macrophages activation in the visceral and subcutaneous adipose tissue of patients with NAFLD correlates with progression from simple steatosis to NASH and fibrosis [71].

Leptin is a 16-kiloDalton anorexigenic hormone with pro-inflammatory actions that prevents lipid accumulation in non-adipose sites; in the liver this is achieved by lowering the expression of SREBP-1 [72]. However, leptin increases in obese subjects as a consequence of leptin resistance and its pro-fibrogenic role has been demonstrated in various in vitro and animal models [73, 74]. Leptin activates hepatic stellate cells through the hedgehog [75] and mTOR [76] pathways. It was suggested that Kupffer cells are targeted by leptin and are stimulated to produce TGF-β1 and subsequently activate hepatic stellate cells [77]. Low-dose gut-derived endotoxin induces hyper-responsiveness to leptin-mediated signaling and subsequent upregulation of CD14 and accelerated fibrosis in NASH mice [78]. In mice models of NASH cirrhosis, hyperleptinaemia aggravates intrahepatic resistance and portal hypertension [79].

Despite the abundance of data in animal models, the effects and significance of serum leptin levels in patients with NAFLD or NASH is still unspecified. [80].

Adiponectin improves hepatic and peripheral insulin resistance and has anti-inflammatory and hepato-protective activities [81]**. These effects are partly achieved by enhancing the deacylation of the sphingolipid ceramide independently of AMPK, especially in hepatocytes, cardiomyocytes and β-cells** [82]. The anti-inflammatory effects are achieved through blocking the activation of NF-kB, secreting anti-inflammatory cytokines and inhibition inhibiting the release of pro-inflammatory cytokines such as TNF-a and IL-6 [83]. Adiponectin also has a direct antifibrotic effect [84], which could be mediated by the activation of AMPK [85]. Enhanced liver fibrosis has been demonstrated in mice lacking adiponectin [84], while delivery of recombinant adiponectin significantly improved steatohepatitis in mice [86]. The antioxidant effects of adiponectin are mediated through its receptor AdipoR1, therefore their decreased levels in obesity may have causal roles in mitochondrial dysfunction and insulin resistance [87]. In obese patients, reduced adiponectin and increased leptin levels may result in hepatic steatosis and activation of inflammation and fibrogenesis [80].

4. Genetic determinants

Genetic variants, especially in the form of single nucleotide polymorphisms, influence hepatic FFAs flux, oxidative stress, response to endotoxins and cytokine production and activity, and are determinants of NAFLD development and progression [88].

Genome-wide association studies have established the role of patatin-like phospholipase 3 (PNPLA3) single nucleotide polymorphisms in the development and progression of NAFLD, in

particular the I148 M (rs738409 C/G) variant. PNPLA3 gene encodes for a protein called adiponutrin which has significant homologies to enzymes implicated in lipid metabolism processes and could exert a lipolytic activity on triglycerides [89].

The PNPLA3 148M allele is associated with lower DNL and expression of the lipogenic transcription factor SREBP1c, despite a substantially increased hepatic fat content, while hepatic β-oxidation is apparently not influenced by the PNPLA3 genetic variant [90]. Furthermore, hepatic lipid accumulation in PNPLA3 148M carriers is associated with decreased secretion of triglyceride-rich lipoproteins from the liver [91].

PNPLA3 I148M knock-in mice accumulate PNPLA3 on lipid droplets and develop hepatic steatosis [92]. In humans, this polymorphism is associated with increased steatosis and fibrosis and is responsible for 5.3% of the total genetic variance attributed to NAFLD [93]. This variant has similar effect size in European and African- and Hispanic-American ancestries, with a variation of frequency in the effect G allele [94].

The above observation was confirmed in a recent meta analysis of 23 studies, where a significant association between the rs738409 PNPLA3 polymorphism and the risk of NAFLD (OR=3.41) and NASH (OR=4.44) was demonstrated [95]. In a case control study of 100 patients with NAFLD-related HCC and 275 NAFLD controls, the G allele conferred an additive risk for HCC (adjusted OR 2.26) [96].

A variant of transmembrane 6 superfamily member 2 (TM6SF2) gene is also possibly involved in NAFLD pathogenesis [24]. TM6SF2 protein promotes VLDL secretion, while the rs58542926 variant, through a loss of function, is associated with hepatic steatosis, lower plasma levels of

VLDL and higher ALT levels [97]. The TM6SF2 minor allele was associated with advanced hepatic fibrosis in two cohorts of NAFLD, comprising 349 and 725 patients (derivation and validation cohort) respectively [98]. Despite of being at higher risk of NASH progression, carriers of this variant are protected against cardiovascular disease, probably due to reduced VLDL levels [99].

5. Epigenetic factors

Clinical studies investigating epigenetic reprogramming in NASH are just beginning to emerge.

Epigenetic modifications are stable changes at transcriptional level, such as DNA methylation, histones modifications and activity of microRNAs (miRNAs), which do not alter the basic DNA sequences and contribute to the cell homeostasis exhibiting a high degree of developmental and environmentally driven plasticity [100]. It has been hypothesized that the disruption of this balance may determine an increased susceptibility for NAFLD [101]. In a study on mice models of diet-induced NASH, an association between epigenetic modifications, particularly pronounced loss of genomic and repetitive sequences cytosine methylation, and the development NASH was observed [102].

DNA methylation is considered as one of the important determinants in the process leading from simple steatosis to NASH and is mostly influenced by dietary deficiency of fundamental methyl donors such as betaine, choline and folate [103]. Supplementation of betaine is associated with a reduced methylation of the MTTP promoter and consequent promotion of triglyceride export from the liver [104]. Folate influences the expression of genes involved in FFA synthesis and its deficiency induce triglyceride accumulation in the liver [105] while feeding mice with a methionine and choline deficient diet results in one of the most common murine

models of NASH, probably caused by the depletion of mitochondrial S-adenosyl-L-methionine and glutathione [106].

Alteration of histone acetylation, mediated by histone deacetylases (HDACs) and histone acetyltransferases (HATs), is another frequent modification and also one of the most studied. Sirtuins (SIRTs, silent information regulator-2 family) form a group of proteins with deacetylase activity; SIRT1 in particular regulates proteins involved in metabolic processes such as glucose homeostasis, oxidative stress, lipid metabolism and inflammatory activity. It also modulates genes such as FXR and LXR and is regulated by JNC [101]. A correlation between reduced expression of SIRT1 or its decreased activity and NAFLD has been demonstrated in both animal models and humans [107, 108].

Epigenetic modifications can be inherited from parents to their offsprings: rats born from mothers fed with high fat diet showed a NAFLD phenotype, as well as DNA hypo-methylation of an hepatic cell cycle inhibitor (Cdkn1) whose subsequent overexpression has been associated to hepatocyte growth in pathological states [109]. Furthermore, high-fat maternal diet in mice correlated with reduced HDAC1 proteins in the fetal liver [61], possibly leading to changes in fetal chromatin [110].

In a study that included morbidly obese patients with all stages of NAFLD and healthy controls, NAFLD-specific expression and methylation differences were seen for nine genes coding for key enzymes in intermediate metabolism and insulin/insulin-like signaling. These NAFLD-specific methylation changes were partially reversible after bariatric surgery. [111].

It has furthermore been demonstrated that non-coding RNAs regulate epigenetic mechanisms of gene expression. Studies on non-coding RNAs in NASH have so far been limited to miRNAs. MiRNAs are small endogenous single-stranded RNA molecules which, by influencing transcriptional and post-transcriptional regulation of gene expression, regulate different cellular processes like proliferation, apoptosis, differentiation and cellular growth [112]. Variations in the expression and circulating levels of miRNAs have been linked to the pathogenesis of NAFLD/NASH: several miRNAs are differentially expressed in subjects with NASH and are associated with glucose and lipid metabolism [113] [114]. MiR-122 is the most abundant miRNA in the liver; studies on animal models have demonstrated that its inhibition leads to reduced plasma cholesterol levels and altered expression of hepatic genes involved in cholesterol and fatty acids synthesis [115]. A human study demonstrated that 113 miRNAs were differentially expressed in the visceral adipose tissue of subjects with NASH as compared to those with simple steatosis: of these, seven were significantly associated to the presence of NASH and were involved in regulation of genes of insulin-related, inflammatory and metabolic pathways, while two (miR-197, miR-99) also correlated with the presence and extent of liver fibrosis [116].

6. Dietary factors

Dietary factors, both in terms of quantity and caloric intake but also of specific nutrients, contribute to the development of NAFLD and NASH. In a study of 18 healthy individuals, doubling the regular caloric intake with fast-food based meals resulted in profound ALT elevations and increased steatosis in less than 4 weeks [117].

Fructose is a lipogenic, pro-inflammatory dietary factor that results in oxidative stress and upregulation of TNF-α [118]. **It is cleared by the liver within first-pass metabolism by 90% and is metabolized by specific hepatic kinases, independently of insulin action, into fructose-1-phosphate. This metabolite is converted into triose phosphate, which enters the glycolytic pathway generating substrates for DNL** [119]. In mice models, fructose-induced NAFLD is associated with bacterial overgrowth and increased intestinal permeability [120]. Fructose can also induce copper deficiency in mice and subsequent NAFLD [121]. In patients with NAFLD, daily fructose ingestion is associated with increased fibrosis [122] and it has been suggested that this is due to industrial rather than fruit-derived fructose [123]. This effect could be mediated by the depletion of hepatic ATP [124].

Sweetened beverages that contain fructose-containing sugars such as sucrose and high fructose corn syrup are associated with increased risk of developing steatosis and NASH, especially in overweight/obese individuals [125].

Conversely, coffee is protective in patients with NAFLD [126]; this action is due to various antioxidants but also to the caffeine per se [127, 128] .

Mono-unsaturated fats, which are typical in a Mediterranean type diet, are protective of NAFLD. In a small 6-week randomized trial, the Mediterranean diet improved steatosis and insulin resistance even without weight loss in patients with insulin resistance and NAFLD [129].

Regarding alcohol, it has been suggested that moderate alcohol consumption is protective in NAFLD. In ob-ob mice, moderate amounts of alcohol, resulted in less steatosis, inflammation and lower transaminases compared to controls: this effect was achieved by activation of

sirtuin1/adiponectin-signaling pathway in the visceral fat tissue and Akt, a key insulin signaling mediator [130]. Conversely, in wild type mice combination of high fat diet and intermittent alcohol consumption was associated with increased steatosis, inflammation and fibrosis [131].

In humans, light to moderate alcohol consumption was associated with reduced incidence of NAFLD in a Japanese study of over 5000 individuals [132]. In a meta-analysis of more than 40,000 individuals, moderate alcohol consumption was associated with reduced prevalence of NAFLD in the general population and of NASH in patients with NAFLD [133]. The above finding will need validation in prospective cohorts.

Influence of the microbiota: the gut-liver axis

It is increasingly recognized that the gut microbiome is implicated in the pathogenesis and progression of NAFLD, through the so-called gut-liver axis. A detailed analysis of the human microbiome has been performed and distinct “enterotypes” are recognized [134]. An obese microbiome has been demonstrated in mice models, which has an increased capacity to harvest energy from the diet [135]. Moreover, this obesity trait is transmissible, as colonization of germ-free mice with the 'obese microbiome' results in a significantly greater increase in total body fat than colonization with a 'lean microbiome' [135].

By receiving more than 50% of its blood supply from the splanchnic district, the liver is one of the most exposed organs to gut-derived toxins and also represents the first line of defense against bacterial-derived products. The cross-talk between host and microbiota at the intestinal mucosal interface is fundamental for the development and homeostasis of the host innate and

adaptive immune system [136]. Lipopolysaccharide (LPS) is one of the main toxic bacterial products and it may act, linked to co-receptor CD14, as ligand for TLR with consequent activation of the inflammatory cascade, including stress-activated protein kinases, JNK, p38, interferon regulatory factor 3 and nuclear factor-jB, pathways which have demonstrated effects on insulin resistance, obesity, hepatic fat accumulation and NASH development and progression [136, 137]. Certain patterns of microbiome diversities can enhance intestinal mucosal permeability and result in lipopolysaccharidemia. Patients with NAFLD have significantly increased gut permeability and a higher prevalence of small intestine bacterial overgrowth compared to healthy controls. [138]. The significant correlation between intestine bacterial overgrowth and NASH is observed even in the absence of increased mucosal permeability [139].

The gut microbiota impact on the host energetic balance by fermenting resistant starch and non-starch polysaccharides to short-chain fatty acids (SCFA), mainly acetate, propionate, and butyrate, and by making them absorbable by the intestinal epithelium [140]. Furthermore, enteric bacteria suppress the synthesis of fasting-induced adipocyte factor (Fiaf) resulting in increased lipoprotein lipase activity and increased triglyceride accumulation [51]. Gut microbiota also produces enzymes that catalyse the conversion of dietary choline into toxic compounds (particularly methylamines). These amines can be uptaken by the liver, transformed to trimethylamine-Noxide and induce inflammation and liver injury [141]. As shown in a recent study, microbiota dysbiosis can promote NASH by either reducing choline levels or increasing methylamines levels [142]. Another mechanism through which gut microbiota promotes NAFLD is the alteration of bile acid metabolism influencing farnesoid X receptor (FXR) signaling and therefore processes such as hepatic DNL and VLDL export [143].

Intestinal microbiota is also the major source of endogenous alcohol production and obesity-associated abnormalities of intestinal microbiome correlate with elevated breath alcohol levels in murine models [144]: in a similar way, a study aiming at identifying possible differences in gut microbiomes of NASH, obese, and healthy children, showed an abundance of alcohol-producing bacteria in NASH microbiomes with a subsequent increase in blood-ethanol concentrations [145]. This hypothesis could explain the similarity of hepatic histological and genetic features that exist between alcoholic and nonalcoholic liver disease.

Finally, defects in the NALP3 inflammasome are associated with lower levels of IL-18 and other cytokines in the intestine, resulting in modulation of the gut microbiome. This is associated with increased influx of TLR4 and TLR9 agonists into the portal circulation and subsequently more severe NASH. Remarkably, co-housing of wild-type mice with mice defective in inflammasome, led to the transmission of a NASH phenotype through the transmission of the altered microbiome [146].

7. Conclusions

The pathogenesis of NAFLD and its progression is a complex process, which cannot be completely explained by the 'two-hit' hypotheses. It is becoming increasingly evident that the different histological patterns of NAFLD, namely simple steatosis and NASH not only have different risk of progression but may also reflect different disease entities in terms of pathogenesis. A number of diverse parallel processes contribute to the development of steatosis and liver inflammation. The gut microbiome has a key role through the gut-liver axis, together with insulin resistance, hormones secreted from the adipose tissue and obesity.

Changes in the gut microbiota as seen in obesity and insulin resistance have consequences both on energy homeostasis and systemic inflammation secondary to endotoxemia. At the liver level, FFAs overflow may result in ER stress and activation of the UPR or mitochondrial dysfunction and consequent activation of inflammatory responses. Furthermore, genetic factors might explain certain more progressive disease courses in NAFLD.

Further characterization of such pathways is important for the development of new non-invasive markers of NASH and fibrosis and for targeted pharmacological treatments.

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Captions

Figure 1. Natural history of NAFLD.

Abbreviations: NAFLD, non-alcoholic fatty liver disease; CVD, cardiovascular disease; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; (% prevalence/incidence); *In 10 years from development of cirrhosis [147].

The term NAFLD encompasses a wide spectrum of conditions, from simple accumulation of fat ('fatty liver' or steatosis) to steatohepatitis (NASH), fibrosis and cirrhosis with its clinical consequences. Despite the high prevalence of NAFLD in the general population, the vast majority of patients have simple steatosis, which is not associated with impaired survival. Only 5-10% of patients diagnosed with NAFLD will develop NASH and 30% of these well develop cirrhosis.

Figure 2. Multiple hit hypothesis for the development of NAFLD

Abbreviations: FFAs, free fatty acids; DNL, de novo lipogenesis; VLDL, very low density lipoproteins; CH, cholesterol; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; TG, triglycerides; ROS, reactive oxygen species; ER, endoplasmic reticulum; UPR, unfolded protein response; LPS, lipopolysaccharide; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

Dietary and environmental factors, together with obesity, lead to raised serum levels of fatty acids (FFAs) and cholesterol (CH), development of insulin resistance, adipocyte proliferation and dysfunction and changes in the intestinal microbiome.

Insulin resistance acts on adipose tissue worsening adipocyte dysfunction, induces lipolysis and release of adipokines and proinflammatory cytokines such as TNF-α and IL-6, which also contribute to maintain the insulin resistance state. In the liver, insulin resistance amplifies DNL.

The increased hepatic FFAs flux which derives from the above processes and from an altered activity of the gut microbiome leads to two different situations: synthesis and accumulation of triglycerides (TG) and 'toxic' levels of fatty acids, free cholesterol and other lipid metabolites which cause mitochondrial dysfunction with oxidative stress and production of ROS and endoplasmic reticulum (ER) stress with activation of UPR, all leading to hepatic inflammation.

Also, small bowel permeability can be enhanced with consequent raised circulating levels of molecules which contribute to the activation of inflammasome and ER stress, such as LPS, and to the release of pro-inflammatory cytokines.

Genetic factors or epigenetic modifications affect hepatocyte fat content, enzymatic processes and liver inflammatory environment, thus influencing the risk of progression to inflammation and fibrosis (NASH) or persistence in a stable stage of disease (NAFLD).