

Review

# Non-Alcoholic Fatty Liver Disease: From Pathogenesis to Clinical Impact

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**Abstract:** Non-Alcoholic Fatty Liver Disease (NAFLD) is caused by the accumulation of fat in over 5% of hepatocytes in the absence of alcohol consumption. NAFLD is considered the hepatic manifestation of metabolic syndrome (MS). Recently, an expert consensus suggested as more appropriate the term MAFLD (metabolic-associated fatty liver disease). Insulin resistance (IR) plays a key role in the development of NAFLD, as it causes an increase in hepatic lipogenesis and an inhibition of adipose tissue lipolysis. Beyond the imbalance of adipokine levels, the increase in the mass of visceral adipose tissue also determines an increase in free fatty acid (FFA) levels. In turn, an excess of FFA is able to determine IR through the inhibition of the post-receptor insulin signal. Adipocytes secrete chemokines, which are able to enroll macrophages inside the adipose tissue, responsible, in turn, for the increased levels of TNF- $\alpha$ . The latter, as well as resistin and other pro-inflammatory cytokines such as IL-6, enhances insulin resistance and correlates with endothelial dysfunction and an increased cardiovascular (CV) risk. In this review, the role of diet, intestinal microbiota, genetic and epigenetic factors, low-degree chronic systemic inflammation, mitochondrial dysfunction, and endoplasmic reticulum stress on NAFLD have been addressed. Finally, the clinical impact of NAFLD on cardiovascular and renal outcomes, and its direct link with type 2 diabetes have been discussed.

**Keywords:** NAFLD; insulin resistance; metabolic syndrome; cytokines; CV risk



**Citation:** Caturano, A.; Acierno, C.; Nevola, R.; Pafundi, P.C.; Galiero, R.; Rinaldi, L.; Salvatore, T.; Adinolfi, L.E.; Sasso, F.C. Non-Alcoholic Fatty Liver Disease: From Pathogenesis to Clinical Impact. *Processes* **2021**, *9*, 135. <https://doi.org/10.3390/pr9010135>

Received: 21 December 2020

Accepted: 8 January 2021

Published: 11 January 2021

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## 1. Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD) is caused by the accumulation of fat in over 5% of hepatocytes in the absence of alcohol consumption [1,2].

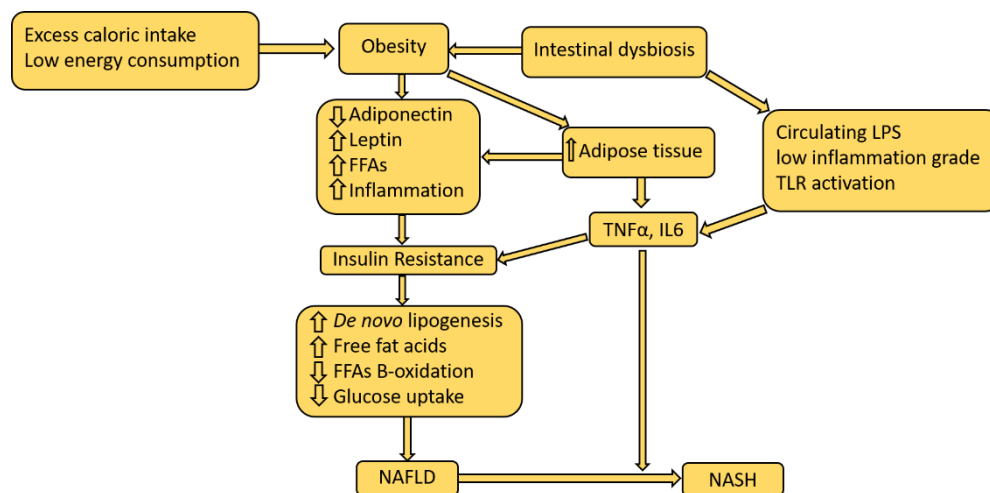
NAFLD clinical presentation is heterogeneous. In fact, it may be represented by hepatic triglycerides accumulation, i.e., hepatic steatosis, or non-alcoholic steatohepatitis (NASH), also characterized by intense necro-inflammatory activity. Both conditions may either regress or evolve into overt liver cirrhosis, with all the related complications (e.g., liver failure, portal hypertension, hepatocellular carcinoma) [3]. To date, NAFLD has become the most frequent cause of chronic liver disease (CLD), while infective forms have been progressively slowing down. Its growing prevalence is closely related to the typical lifestyle changes of the Western culture, i.e., a trend towards overeating and a sedentary lifestyle. NAFLD, indeed, is considered the hepatic manifestation of metabolic syndrome (MS), even though its etiology could involve genetic factors and could also affect lean and non-diabetic people [4–7]. The pathophysiologic basis of MS, i.e., insulin resistance (IR), also plays a crucial role in NAFLD pathogenesis too [8], which has led to the consideration

of a more appropriate term—MAFLD (metabolic associated fatty liver disease)—to replace NAFLD [9].

The aim of this review is to summarize the pathophysiological mechanisms of NAFLD development and its close link with insulin resistance. We will also focus on the clinical impact of NAFLD on both cardiovascular and renal outcomes, and type 2 diabetes.

## 2. Pathophysiological Mechanisms of NAFLD

Historically, NAFLD and its progression towards NASH have been attributed to the so-called “two-hits hypothesis” [10]. However, most recent evidence has suggested more complex mechanisms, named as the “multiple parallel hits hypothesis” theory, which involves different elements acting together, rather than in series [11]. According to this theory, IR, genetic and epigenetic factors, mitochondrial dysfunction, endoplasmic reticulum stress, microbiota, chronic low-grade inflammation, and dysfunction of adipose tissue all represent synchronic causes of both NAFLD development and progression [12] (Figure 1).



**Figure 1.** Schematic representation of the pathophysiological mechanisms of Non-Alcoholic Fatty Liver Disease (NAFLD) development. LPS: lipopolysaccharides; TNF- $\alpha$ : tumor necrosis factor-alpha; IL-6: interleukine-6; TLR: toll-like receptor; FFAs: free fatty acids.

## 3. Insulin Resistance

Insulin resistance (IR) is a condition in which insulin action is altered. In metabolic terms, IR represents the inability of a fixed amount of insulin to metabolize a known amount of glucose in an individual as compared to the general population.

IR is closely related to visceral fat and represents the cornerstone of metabolic syndrome (MS), which is defined by the presence of at least three among the following features: abdominal obesity, increased triglycerides levels, reduced HDL cholesterol levels, increased blood pressure, and hyperglycemia [13].

Insulin hormone is produced by the beta cells of the pancreas [14]. Once secreted, it binds to the extracellular domain of its specific receptor, thus causing a conformational change, with the subsequent phosphorylation of specific tyrosine residues on the intracytoplasmic domain of the receptor itself [15]. Then, the activated receptor hooks the so-called insulin receptor substrates (IRS), which are in turn activated by tyrosine-phosphorylation processes. This cascade of events triggers a series of downstream phosphorylation inside the cell, which thus becomes able to mediate the effects of insulin. In detail, IRS molecules become able to activate phosphatidylinositol 3-kinase (PI3K), thus causing the translocation of glucose transporter type-4 (GLUT-4) protein from the cytoplasm to the cytoplasmic membrane. In this way, GLUT-4 is able to capture glucose from the bloodstream and internalize it inside the cell. In addition to the crucial role of enabling intracellular glucose entry,

activated PI3K may also trigger anti-lipolytic effects, activation of fatty acid synthase, and glycogen synthase, thus mediating the typical anabolic effects of insulin. Phosphorylated IRS also leads to the activation of the Ras/MAPK pathway, involved in cell survival and stimulation of mitosis [16]. This suggests that the two biochemical cascades activated by insulin, i.e., the regulation of intermediate metabolism and the stimulation of cell growth and proliferation, might be dissociated in their regulation.

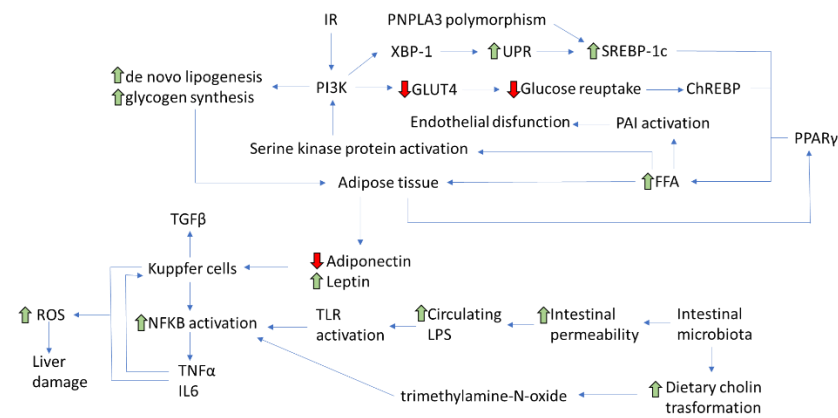
The complexity of the insulin metabolic pathway explains why several interferences may affect its biochemical signal. On the other hand, IR may be mediated by alterations at any level of its biochemical signal cascade. IR rising during MS is characterized by the impact of glucose on cellular absorption processes, by the suppression of lipolysis of adipose tissue, and the impairment of vasodilation, whilst action on growth and cytogenesis are not affected [17]. The selective inhibition of insulin action on metabolism and vasodilation might be explained by interference at IRS and PI3K [18].

Genetically induced alterations triggering IR are heterogeneous and may affect the bond between insulin and its receptor, the autophosphorylation process, and the kinase activity of either IRS or PI3K or MAP kinase [19,20]. However, IR during MS is mainly determined by environmental factors due to an improper lifestyle, i.e., a discrepancy between caloric intake and energy consumption. As a consequence, the development of visceral obesity, a risk factor for the onset of both T2DM and cardiovascular (CV) diseases, is the result of this positive energy balance [21,22]. The flow of fatty acids originating from the visceral adipose tissue determines its pathophysiologic effects [23], thus establishing a direct correlation between IR and the amount of visceral adipose tissue mass [24]. In fact, the action of insulin is mainly related to the visceral adipose tissue rather than to the subcutaneous adipose tissue. In this regard, weight loss has been proven to improve insulin sensitivity, as well as its association with changes in the mass of visceral adipose tissue, and of either total or subcutaneous adipose tissue [25,26].

Metabolic regulation of the neuroendocrine axis includes adipose tissue, alongside the central nervous system (CNS) and intestine, through the regulation of insulin sensitivity in the target tissues [27]. Adipocytes store and release fatty acids in the form of triglycerides according to the body's needs. Target tissues (e.g., the skeletal muscle) may oxidize fatty acids and use them as fuel. However, elevated levels of circulating fatty acids can desensitize target tissues to the actions of insulin.

Adipocytes also secrete other hormones: adipokines. Among them, adiponectin acts as a stimulator of insulin action in the peripheral tissues [28], whilst leptin and resistin inhibit the sensitivity to insulin action [29]. In the presence of visceral obesity, leptin levels increase whilst adiponectin reduces. Moreover, adipocytes secrete chemokines, which are able to enroll macrophages inside the adipose tissue, responsible, in turn, for the increased levels of tumor necrosis factor-alpha (TNF- $\alpha$ ) [30]. The latter, as resistin and other pro-inflammatory cytokines such as interleukin-6 (IL-6), enhance the insulin resistance phenomena and correlates with the endothelial dysfunction degree [31].

As aforementioned, the increase in the mass of visceral adipose tissue also determines, besides the imbalance of adipokine levels, an increase in free fatty acids (FFA) levels. In turn, an excess of FFA is able to determine IR through the inhibition of the post-receptor insulin signal. Indeed, the increase in FFA determines the activation of a serine kinase protein able to phosphorylate the serine residues on IRS and PI3K molecules [32]. Then, IRS and PI3K can no longer be phosphorylated at the level of their tyrosine residues and thus cannot be adequately activated, with the subsequent result of a block in the transmission of insulin signal [32]. Similar effects are also caused by elevated levels of TNF- $\alpha$  and IL-6 [33]. The increase in plasma circulating triglycerides also determines an elevated synthesis of plasminogen activator inhibitor-1 (PAI-1) by endothelial cells, supporting the subsequent endothelial dysfunction and the clinical development of arterial hypertension [34]. Therefore, an extremely positive caloric balance due to an unhealthy lifestyle causes IR by the increase in visceral adipose tissue and the consequent release of FFA, TNF- $\alpha$ , and adipokines (Figure 2).



**Figure 2.** Main pathophysiological pathways involved in NAFLD development (IR: Insulin resistance; PNPLA3: patatin-like phospholipase domain-containing protein 3; XBP-1: X-box Binding Protein-1; UPR: Unfolded Protein Response; SREBP-1: sterol regulatory element-binding protein-1; PI3K: phosphatidylinositol 3-kinase; GLUT-4: glucose transporter type-4; ChREBP: carbohydrate-responsive element-binding protein; PAI-1: plasminogen activator inhibitor-1; PPAR- $\gamma$ : peroxisome proliferator-activated receptor-gamma; FFA: free fatty acids; TGF- $\beta$ 1: transforming growth factor-beta 1; TNF- $\alpha$ : tumor necrosis factor-alpha; NF- $\kappa$ B: nuclear factor kappa-light-chain-enhancer of activated B cells; TLR: toll-like receptors; LPS: lipopolysaccharide; IL6: interleukin -6; ROS: Reactive Oxygen Species).

### *Insulin Resistance and NAFLD Development*

IR plays a key role in the development of NAFLD, as it causes an increase in hepatic lipogenesis and an inhibition of adipose tissue lipolysis, with a subsequent elevated flow of fatty acids in the liver [35,36]. Fat accumulates within the hepatocytes mainly as triglycerides deriving from the esterification of glycerol and FFAs [37,38]. The hepatic accumulation of triglycerides does not represent itself as a hepatotoxic event, rather as a defense mechanism able to balance the excess FFAs in the plasma [39]. However, other bioactive intermediates, such as ceramides and diacylglycerol (DAG), can induce lipotoxicity, resulting in inflammation, necrosis, and liver fibrosis. NAFLD progresses to NASH when the mechanisms protecting hepatocytes from lipotoxicity are depleted. This induces necrosis and secondary repair phenomena mediated by hepatic stellate cells, with the deposition of scar collagen tissue, hence the development and progression of fibrosis [40].

As already described, NAFLD patients display a de novo hepatic lipogenesis higher than healthy controls, which is neither suppressed in fasting nor with higher FFA plasma levels [41]. Hepatic lipogenesis during IR can be further induced by the activation of transcription factors, such as the sterol regulatory element-binding protein-1 (SREBP-1), the carbohydrate-responsive element-binding protein (ChREBP), and the peroxisome proliferator-activated receptor-gamma (PPAR- $\gamma$ ) [42]. SREBP-1 is a transcription factor present in different isoforms: SREBP-1c, which regulates de novo lipogenesis and is stimulated by insulin, and SREBP-2, instead involved in cellular cholesterol homeostasis [43]. ChREBP is activated by glucose and induces lipogenesis, but it also provides more substrates for the synthesis of both triglycerides and FFA. Among insulin receptors, IRS-2, when activated, can act as a regulator of SREBP-1c, thus affecting de novo lipogenesis [44]. Under IR conditions, IRS-2 is downregulated, with overexpression of SREBP-1c resulting in a stimulation of lipogenesis [45]. At the same time, FFA beta-oxidation is inhibited, which further favors the hepatic accumulation of lipids [46]. In addition, insulin has a powerful suppression action on adipose tissue lipolysis, usually compromised during IR, resulting in an increase in the flow of FFA to the liver [47]. Once accumulated in the liver, FFAs induce alterations in the insulin signaling pathways through the activation of serine kinase and contribute to the worsening of the systemic state of IR [48]. Conversely, NAFLD itself contributes to the development of IR, leading to the establishment of a vicious circle. In fact, in NAFLD patients, genetic and environmental factors can interfere with the insulin post-receptor signal cascade through the phosphorylation of serine residues on

insulin [49], the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), and the Suppressor Of Cytokine Signaling-3 (SOCS-3) [50]. Therefore, the relationship between IR and NAFLD seems bidirectional. Indeed, the complex network of events involved in the development of NASH may determine an increase in hepatic IR up-regulating SOCS-3 which, through the interference with IRS-1, determines a reduced insulin signal transmission [51,52]. Therefore, it seems likely that an improvement in IR degree could help to reduce NAFLD progression and should thus be considered among the main therapeutic targets (Figure 2).

Intriguingly, an old drug that always comes in handy is metformin which, by ameliorating IR as well as several other mechanisms, is able to determine important beneficial changes in the global risk profile [53–55].

#### 4. Role of Visceral Adipose Tissue

As aforementioned, adipose tissue exerts an endocrine function by secreting hormones, known as adipokines, such as leptin and adiponectin [56]. Adipocyte hypertrophy secondary to visceral obesity and IR causes an imbalance in the production of adipokines, which, in turn, affects the adipose tissue and the liver [57,58]. Leptin is an anorectic hormone with a pro-inflammatory action that prevents the accumulation of lipids in ectopic sites. In the liver, this effect is produced by a reduced expression of SREBP-1 [59]. In obese individuals, we observe elevated levels of leptin due to leptin-resistance, with a consequent exacerbation of inflammation and liver fibrosis [60]. In fact, Kupffer cells are stimulated by leptin to produce transforming growth factor-beta 1 (TGF- $\beta$ 1) and activate hepatic stellate cells [61] by “hedgehog” [62] and mTOR [63] pathways. Overall, these events contribute to liver fibrosis and, eventually, to cirrhosis.

On the contrary, adiponectin improves both liver and peripheral resistance to insulin and exerts an anti-inflammatory and hepato-protective role [64]. The anti-inflammatory effects depend on several mechanisms: the block of NF- $\kappa$ B activation, the production of anti-inflammatory cytokines, and the inhibition of the release of proinflammatory cytokines such as TNF- $\alpha$  and IL-6 [65]. Adiponectin also exerts a direct antifibrotic effect mediated by the activation of adenosine monophosphate-activated protein kinase (AMPK) [66]. Finally, this adipokine also displays antioxidant effects, which seems mediated by its receptor AdipoR1. Therefore, reduced levels of adiponectin during obesity may result in mitochondrial dysfunction and insulin resistance [67]. As a whole, the reduced levels of adiponectin and the increased levels of leptin in patients with MS can induce steatosis and liver inflammation, thus activating fibrogenesis [68].

As later discussed, visceral adipose tissue can cause NAFLD and NASH, also inducing a chronic inflammatory state.

#### 5. Role of Diet

It is well known that both quantitative (caloric intake) and qualitative (the type of nutrients introduced) characteristics of the diet may be involved in the development of NAFLD as well as in the evolution towards NASH [7]. Overeating is clearly related to hepatic steatosis and to the subsequent risk of steatohepatitis [69,70], but some food substrates are more steatogenic than others. As an example, fructose is a pro-inflammatory lipogenic factor able to cause oxidative stress and TNF- $\alpha$  overproduction [71]. Fructose is almost completely metabolized to fructose-1-phosphate, a metabolite entering the glycolysis metabolic pathway and generating substrates for de novo lipogenesis [72]. A close association was also observed between fructose-induced NAFLD and either bacterial proliferation and increased intestinal permeability [73] and, in NAFLD patients, daily fructose ingestion and increased liver fibrosis are also related [74], a mechanism potentially mediated by the depletion of hepatic ATP [75]. High-calorie drinks contain huge amounts of sucrose, hence fructose and their consumption is associated with an increased risk of developing liver steatosis and NASH [76]. On the contrary, coffee seems to exert a hepato-protective effect in patients with NAFLD [77], more likely due to the various antioxidants and to caffeine

itself, which would have protective properties against liver inflammation [78]. Likewise, monounsaturated fats (typical of the Mediterranean diet) have shown beneficial effects in reducing the degree of IR and improving NAFLD [79,80]. Although these data seem controversial, moderate alcohol consumption has also been suggested to exert a protective role against NAFLD [81].

## 6. Role of Intestinal Microbiota

Increasing evidence suggests that gut microbiota may be involved in both pathogenesis and progression of NAFLD [82–86]. In humans, there are several microbiota enterotypes [87]. A microbiota defined as “obese”, i.e., with a higher ability to absorb energy from the diet, seems able to determine a significantly higher increase in total body fat as compared to an individual colonized by the so-called “lean” microbiota [82]. The intestinal microbiota, indeed, modifies the energy balance of the host through the fermentation of resistant starch and non-starch polysaccharides in short-chain fatty acids (SCFA), thus promoting their absorption by the intestinal epithelium [88].

Beyond the accumulation of fatty acids inside the hepatocytes, intestinal microbiota may also promote inflammation and favor the mechanisms of liver damage progression. In fact, over 50% of the blood supply in the liver is provided by the splanchnic district, thus being among the organs most exposed to toxins of intestinal origin, as it represents the first line of defense against products of bacterial origin. The crosstalk between host and microbiota along the intestinal mucosal interface is fundamental for both the development and homeostasis of the host’s innate and adaptive immune system [89]. Patients with NAFLD show a significantly higher intestinal permeability and a larger bacterial proliferation of the small intestine as compared to healthy controls [83,84]. Lipopolysaccharide (LPS) is among the most toxic products of bacterial derivation and can act as a toll-like receptor (TLR) ligand, with consequent activation of the inflammatory cascade and action on insulin signal, obesity, liver fat accumulation, and development and progression towards NASH (Figure 2) [90].

The intestinal microbiota is also able to produce enzymes that catalyze the conversion of dietary choline into toxic compounds (e.g., methylamines), which may reach the liver. Inside the liver, they are transformed into trimethylamine-N-oxide, able to induce inflammation and hepatic damage [91]. Microbiota dysbiosis can thus promote NASH by reducing choline levels and increasing methylamine levels [85,92].

Finally, other mechanisms seem to be involved in NAFLD promotion through the intestinal microbiota. Interestingly, the latter seems able to alter the metabolism of bile acids, affecting de novo lipogenesis and VLDL export processes [86]. Moreover, the gut microbiota also represents the main source of endogenous alcohol production and its abnormalities associated with obesity are related to high alcohol levels [93]. This hypothesis might explain the similar hepatic histological and genetic characteristics between alcoholic and non-alcoholic forms of liver steatosis.

## 7. Role of Genetic and Epigenetic Factors

Genetic variants, especially single nucleotide polymorphisms, can affect several processes: FFAs flow into the liver, oxidative stress, response to endotoxins, production and activity of cytokines. Thus, they seem crucial both in the development and progression of NAFLD [94]. Several studies have established the role of single nucleotide polymorphisms of the patatin-like phospholipase domain-containing protein 3 (PNPLA3) in NAFLD development and progression, particularly the variant I148M (rs738409 C/G) [94–97]. PNPLA3 gene encodes a protein named adiponutrin, which exerts a lipolytic activity on triglycerides [95]. The PNPLA3 148M allele is associated with a reduced de novo lipogenesis and a higher expression of SREBP-1c [95]. In humans, this polymorphism is further associated with an increased incidence of steatosis and a higher degree of liver fibrosis [95,96]. The association with PNPLA3 polymorphism seems significant also in patients with concomitant causes of liver damage (e.g., HBV or HCV infection) [96]. A meta-analysis including

23 studies confirmed the significant association between PNPLA3 polymorphism and a higher risk of NAFLD and NASH [97]. Even a variant of the TM6SF2 gene is more likely involved in the pathogenesis of NAFLD. The protein resulting from the translation of this gene promotes the secretion of VLDL, while its variant rs58542926, through a loss of function, is associated with lower plasma VLDL levels, hepatic steatosis, and higher ALT levels [98]. Interestingly, this variant's carriers, though presenting a higher risk of NAFLD progression, are burdened by an overall lower CV risk than controls, more likely due to reduced VLDL levels [99].

Beyond the role of genetic variants, some studies have been focusing on the potential impact of epigenetics on the development of NAFLD. Epigenetic modifications represent stable transcriptional changes, such as DNA methylation, histone modifications, and microRNA (miRNA) activity. They do not alter the basic DNA sequences, but they are able to modify their translation. Epigenetics contributes to cellular homeostasis through a high degree of evolutionary plasticity driven by environmental changes [100]. The disruption of this equilibrium has been hypothesized to increase susceptibility to NAFLD [101].

DNA methylation is among the crucial determinants which lead to simple steatosis up to NASH and it is mainly affected by the dietary shortage of methyl group donors (e.g., betaine, choline, and folate) [102]. In fact, large evidence has been provided about the association between betaine dietary supplementation and reduced methylation of the MTP promoter, with the subsequent promotion of the export of triglycerides from the liver [103].

Folate levels affect the expression of the genes involved in FFA synthesis and its deficiency seems involved in the intrahepatocyte accumulation of triglycerides [104]. Sirtuins (SIRT, the family of the silent information regulator-2) are a group of proteins with deacetylase activity. In particular, either a reduced expression of SIRT1 or its reduced activity seem associated with the incidence of NAFLD both in animal models and in humans has been shown [105].

Non-coding microRNAs (miRNAs) have also been shown to regulate epigenetic gene expression mechanisms. MiRNAs are small endogenous single-stranded RNA molecules that, through transcriptional and post-transcriptional regulation of gene expression, regulate several cellular processes (e.g., proliferation, apoptosis, differentiation, and cell growth) [106]. Changes in miRNA expression and circulating levels have been associated with both NAFLD and NASH pathogenesis. Different miRNAs are expressed in individuals with NASH and are associated with glucose and lipid metabolism alterations [107]. MiR-122 is the miRNA mostly expressed in the liver and its inhibition leads to decreased plasma cholesterol levels and an altered expression of liver genes involved in the synthesis of cholesterol and fatty acids [108]. A study has demonstrated how many different miRNAs are activated in the visceral adipose tissue of subjects with NASH as compared to those with simple steatosis, potentially being associated with inflammation and liver fibrosis [109].

## 8. Role of Low-Degree Chronic Systemic Inflammation

Several factors contribute to the production and the release of pro-inflammatory cytokines in MS. In particular, the increased levels of FFA, lipotoxicity, IR, dysfunction of peripheral adipose tissue, and endotoxemia secondary to elevated intestinal permeability are able to induce a low-degree chronic inflammatory state, which seems relevant in the pathophysiological mechanisms of NAFLD and NASH.

Two main inflammatory pathways, JNK-AP-1 and IKK-NF- $\kappa$ B, are critically involved in the development of the chronic inflammation occurring during NAFLD [110]. Jun N-terminal kinase (JNK) is a member of the mitogen-activated protein kinases associated with the induction of apoptosis and the development of NASH. The nuclear factor NF- $\kappa$ B is a transcription factor and a regulator of inflammation, and the IKK2 subunit is the main component required for its activation during the acute inflammatory response [111]. Patients affected by NASH display a persistent activation of the NF- $\kappa$ B pathway [112] and

its persistent activation and the overexpression of IKK2 in hepatocytes lead to a state of chronic inflammation and exacerbate IR [113].

Hepatic exposure to increased levels of pro-inflammatory cytokines leads to histological changes typical of NASH, such as hepatocyte necrosis and apoptosis, neutrophil chemotaxis, activation of hepatic stellate cells, and production of Mallory bodies [114,115]. In fact, obese patients show higher serum levels of TNF- $\alpha$  and IL-6 which, on the other hand, decrease after weight loss [116]. Moreover, patients who develop NASH show higher serum and hepatic TNF- $\alpha$  levels, which are associated with the histological severity of liver damage [117]. The increased expression of inflammatory genes and macrophage activation in visceral and subcutaneous adipose tissue of NAFLD patients are associated with the progression from steatosis to NASH and liver fibrosis [118]. Furthermore, inflammation and activation of NF- $\kappa$ B can promote carcinogenesis, hence the chronic inflammatory state of NAFLD may play a role in HCC development (Figure 2) [119,120].

### 9. Role of Mitochondrial Dysfunction and Endoplasmic Reticulum Stress

All structural and functional alterations in the mitochondria (i.e., the depletion of mitochondrial DNA, morphological and ultrastructural changes and alterations in the respiratory chain and beta-oxidation), contribute to the pathogenesis of NAFLD [121]. In fact, if the mitochondrial and peroxisomal functions are not able to manage the increase in lipid flow, respiratory oxidation collapses, thus leading to impairment in lipid homeostasis, generation of toxic metabolites, and overproduction of reactive oxygen species (ROS) [122,123]. These molecules determine oxidative stress and contribute to hepatic necro-inflammatory processes and the worsening of mitochondrial damage. Indeed, IR, obesity, and TNF- $\alpha$  levels have been proven to be directly associated with mitochondrial dysfunction [124]. In addition, ROS, along with oxidized LDL particles, can activate Kupffer and hepatic stellate cells, thus resulting in the addition of collagen and secondary liver fibrosis [125].

Moreover, increased protein synthesis, endoplasmic reticulum dysfunction, and ATP deficiency may represent the causes of an accumulation of unfolded proteins inside the endoplasmic reticulum. This accumulation activates the unfolded protein response (UPR), an adaptive response to reduce protein synthesis, increase capacity for protein trafficking through the endoplasmic reticulum (ER), protein folding and transport, and increase protein degradative pathways [126]. In the case of failure in solving the protein-folding defect, UPR may induce hepatocytes apoptosis.

The ubiquitin proteasome system (UPS), the major non-lysosomal intracellular protein degradation pathway, can be affected by oxidative stress, thus playing a crucial role in the activation of NF- $\kappa$ B.

Factors inducing UPR during NAFLD include hyperglycemia, mitochondrial damage (and consequent ATP depletion), hypercholesterolemia, phosphatidylcholine depletion, and oxidative stress [127]. UPR in turn induces the activation of JNK, which is able to determine the inflammatory state and the apoptosis involved in the progression of NASH [128], as well as in the impairment of insulin signaling and the subsequent T2DM development [129]. Moreover, UPR also activates SREBP-1c, with a consequent worsening of the accumulation of hepatic fat and the further exacerbation of ER stress [130]. X-box Binding Protein-1 (XBP-1) is among the main regulators of UPR and interacts with the insulin signaling pathway via PI3K [131]. PI3K and XBP-1 interaction both modulate the cell response to endothelial stress and, conversely, the response itself modulates these factors [132], therefore representing a potential link between liver steatosis, IR, and liver inflammation [133]. In this regard, the deregulation of the ubiquitin-proteasome has been proposed as a pathogenetic factor inducing atherogenesis processes and promoting plaque vulnerability in the setting of diabetic patients [134].



## 10. Clinical Impact of NAFLD

### 10.1. NAFLD, CV Disease, and Atherosclerosis

NAFLD is associated with an increased incidence of CV morbidity and mortality [135]. A recent meta-analysis showed that the prognosis of patients with NAFLD is worsened by a rise in the incidence of both fatal and non-fatal CV events [136]. These findings also seem related to the severity of biopsy-demonstrated hepatic fibrosis [137]. NAFLD is also associated with an augmented prevalence of classic CV risk factors (diabetes, visceral obesity, arterial hypertension) [138], though growing evidence has shown an independent association between NAFLD and CV disease [135]. Patients with NAFLD have a growing incidence of both coronary heart disease and mortality from CV events as compared to the general population [135,139]. The RISC study demonstrated that NAFLD was independently associated with an increased 10-year coronary heart disease risk score [140]. A higher prevalence of coronary, cerebrovascular, and peripheral vascular disease has been also detected in patients with T2DM and NAFLD when compared to those without NAFLD [141].

The role of NAFLD in determining subclinical atherosclerosis seems independent of other metabolic factors [142]. As compared to controls, patients with NAFLD show a higher prevalence of angiography demonstrated coronary stenosis [143]. Moreover, in these patients, coronary atheromatic lesions are characterized by more vulnerable plaques, independently of the presence of MS [144]. Likewise, NAFLD has been associated with increased carotid artery intima-media thickness (IMT), arterial wall stiffness, and impaired endothelium-dependent flow-mediated vasodilatation [145–147]. In addition, the risk of progression of coronary and/or carotid atherosclerosis is higher in patients who have a higher NAFLD severity assessed by clinical scores (i.e., NAFLD fibrosis score) [148]. The ultrasound has demonstrated that NAFLD resolution seems to reduce the risk of progression of carotid atheroma [149]. However, due to the tight link between NAFLD and IR, the latter represents one of the major confounders when assessing CV disease development, as it is a well-known risk factor in both primary and secondary prevention [150–152]. In fact, the role of IR in CVD development has been widely assessed by both mathematical and human models and enforced by metanalysis [153–156].

A link with heart failure has been suggested, too. NAFLD patients have a high prevalence of left diastolic dysfunction, left ventricular hypertrophy, and valve diseases such as aortic stenosis [157]. An Italian study showed among patients admitted due to acute heart failure, that NAFLD and its severity were independently associated with increased in-hospital and post-discharge all-cause mortality in the elderly [158]. Moreover, in patients with preserved ejection fraction heart failure, a high NAFLD fibrosis score was associated with a worse outcome [159]. Finally, a large meta-analysis showed a significant association between NAFLD and arrhythmias (particularly atrial fibrillation) [160].

NAFLD is also associated with a higher risk for cerebrovascular events, and the risk of stroke seems independently associated with the degree of hepatic fibrosis [161]. Moreover, it might be associated with more severe stroke (National Institutes of Health Stroke Scale) and worse outcome (Modified Rankin Scale score) [162].

### 10.2. NAFLD and CKD

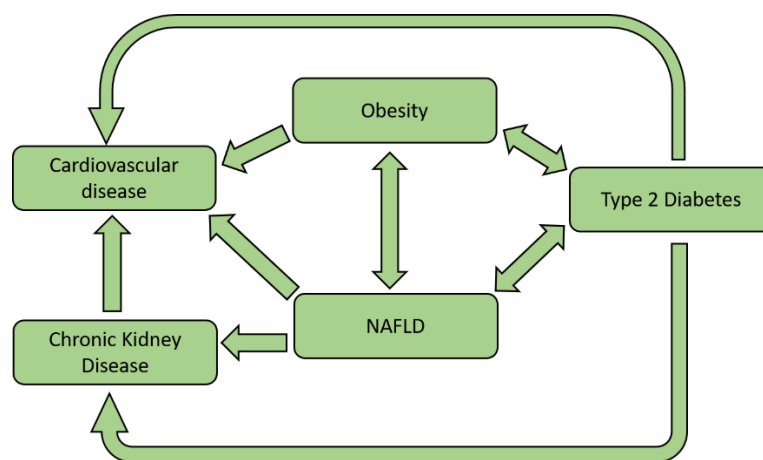
Even though NAFLD and chronic kidney disease (CKD) share several risk factors, numerous evidence suggest that NAFLD ultimately represents an independent risk factor for CKD [163]. In a large meta-analysis, Musso et al. demonstrated an increased prevalence of CKD in NAFLD patients, regardless of the presence of other risk factors (sex, age, duration of diabetes, blood pressure, and smoking) [164]. More recent works confirmed these data and they also allowed to quantify the increase in the long-term risk of CKD to about 40% [163,165–168].

## 11. NAFLD and Type 2 Diabetes

The prevalence of NAFLD in patients with T2DM is extremely varying depending on the screen method used, ranging from 29.6% to 87.1% [169–171]. The role played by hyperglycaemia and IR in the development of NAFLD and fibrosis is well established. In fact, high plasma glucose levels induce toxicity and activate the apoptosis pathway in the liver [172]. Moreover, a study showed a linear correlation between HbA1c and liver fat content [173].

The copresence of both type 2 diabetes and IR determines a worse CV disease risk [174–177]. A similar finding was shown by a recent meta-analysis on NAFLD patients with concomitant type 2 diabetes. In fact, in this population, the CV disease risk was increased by two-fold compared to patients without NAFLD [178].

Beyond cardiovascular outcome and disease progression, the link between NAFLD and diabetes also affects the renal outcome (Figure 3). In fact, the Valpolicella Heart Diabetes Study followed up to six years patients affected by NAFLD and type 2 diabetes with preserved kidney function. Patients affected by NAFLD were burdened by an increased incidence of CKD independent of sex, age, blood pressure, duration of diabetes, and smoking [165]. Moderate to severe CKD have been also independently associated with increased mortality in NAFLD patients, as assessed by The Third National Health and Nutrition Survey database during a long period of follow-up (mean follow-up: 19,2 years) [179]. The presence of diabetic nephropathy has already been identified as a possible marker for patients at particularly high CV risk [180,181], which requires intensive management of all risk factors [182]. In fact, a Swedish study on patients with NAFLD diagnosed by biopsy found that all-cause death was more likely due to CVD secondary to diabetes than CKD during a long period follow-up (mean follow-up: 18,8 years) [183].



**Figure 3.** Schematic representation of NAFLD associated cardiorenal disease and type 2 diabetes development.

Furthermore, it would be certainly clinically useful to assess the correlation between NAFLD and the numerous neurological and sensorineural complications of diabetes [184].

## 12. Conclusions

NAFLD is the leading cause of chronic liver disease, with complex pathophysiology, mostly linked to metabolic disorders, particularly to IR.

The relationship between NAFLD and IR is bidirectional, with one causing or worsening the other and vice versa. The clinical implications associated with these two conditions are an increased risk of CV events, the progression of liver and renal damage, and end-stage renal failure, with a significant impact on both CV and all-cause mortality. IR plays a central role in NAFLD pathophysiology, thus representing a crucial target in the therapeutic strategies.

While we are still waiting for new drugs effective on NAFLD, lifestyle changes come in handy. Currently, weight loss and physical activity, through insulin sensitivity improvement, are able to break the pathophysiological chain, decreasing metabolic substrate delivery to the liver [185].

**Author Contributions:** Conceptualization, F.C.S. and R.N.; writing—original draft preparation, A.C. and C.A.; writing—review and editing, P.C.P., R.G., R.N., L.R., T.S., L.E.A. and F.C.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

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