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Review article

# Heterogeneity of non-alcoholic fatty liver disease (NAFLD): Implication for cardiovascular risk stratification

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ABSTRACT

NAFLD is currently considered the most common liver disease worldwide and mounting data support its strong link with atherosclerotic cardiovascular disease (ASCVD). This association is important as cardiovascular disease (CVD) is generally recognized as the leading cause of death in individuals with NAFLD. However, NAFLD represents a heterogeneous condition showing a wide spectrum of clinical and pathophysiological sub-phenotypes with different adverse outcomes ranging from ASCVD to liver damage progression.

The contribution to NAFLD pathogenesis of different environmental, metabolic, and genetic factors underlies this heterogeneity. The more frequent phenotype of NAFLD patients is associated with metabolic dysfunctions such as obesity and insulin-resistant syndrome and this has been recently named as Metabolic Associated Fatty Liver disease (MAFLD). However, NAFLD is encountered also in subjects without insulin resistance and metabolic alterations and in whom genetic factors play a major role. It has been suggested that these individuals are at risk of liver disease progression but not of cardiovascular complications. Separating metabolic from genetic factors could be useful in disentangling the intricate relationship between NAFLD and atherosclerosis.

In the present review, we aim to address the epidemic of NAFLD, its epidemiologically association with ASCVD complications and the overall mechanisms involved in the pathophysiology of atherosclerotic vascular damage in NAFLD patients. Finally, we will revise the potential role of genetics in identifying disease subtyping and predicting individualised CVD risk.

## 1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease and affects 17%–51% of the adult population worldwide [1–3]. Its prevalence is estimated to continuously increase in parallel to the growth of obesity and diabetes incidence [4]. In 2016, 85.3 million people were estimated to have NAFLD in the United States, and this number is expected to reach 100.9 million in 2030. In parallel with the increase of NAFLD prevalence, its liver complications are estimated to increase by 63% for non-alcoholic steatohepatitis (NASH), 168% for decompensated cirrhosis and 137% for hepatocarcinoma (HCC) [5]. Finally, to date NAFLD is the second most common cause for liver transplantation, and it will probably become the first one soon [6].

Overall, atherosclerotic-related cardiovascular complications are the leading cause of morbidity and mortality in NAFLD patients being 6-fold more frequent than those related to liver causes (incident rate of 4.79 *vs.* 0.77 per 1000 person-years) [2,7,8]. Moreover the annual incidence of ASCVD in NAFLD patients is around 1.1% [9] and considering the spread of the disease, the absolute number of these events is destined to grow significantly [10].

Many studies have been published on the association between NAFLD and CVD risk and their results have been summarised in several meta-analyses, as shown in Table 1. In comparison to patients without NAFLD, those with NAFLD have an increased risk to develop acute myocardial infarction by 65%, stroke by 55% and CKD by 53% [11]. Not least, up to 80% of non-diabetic NAFLD patients will develop impairing fasting glucose or diabetes during their life [12]. Notably, these associations were independent from the standard cardiovascular risk factors,

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**Table 1**
Metanalyses on the association between NAFLD and CVD risk.

| Reference | Characteristics | Outcome | OR* or HR° |
|---|---|---|---|
| [9] | 16 longitudinal studies | Fatal/Non-fatal CV event | 1.64* [1.26–2.13] |
| | 34,000 patients followed-up | CV mortality for advanced liver disease | 3.28* [2.26–4.77] |
| | 7 years | | |
| [14] | 13 prospective studies | CV mortality | 1.10° [0.86–1.4] |
| | 21 cross-sectional | CV incidence | 1.37° [1.10–1.72] |
| | 164,494 patients | CAD incidence | 2.31° [1.46–3.65] |
| [15] | 11 studies | CVD risk T2DM vs NGT NAFLD | 2.20* [1.67–2.90] |
| | 8,342 NAFLD individuals | Increased IMT | 1.74* [1.47–2.06] |
| | | Increased arterial stiffness | 1.56* [1.24–1.96] |
| | | Increased coronary artery calcification | 1.40* [1.22–1.60] |
| | | Endothelial dysfunction | 3.73* [0.99–14.09] |
| [13] | 36 longitudinal studies | Fatal or non-fatal CV events | 1·45° [1·31–1·61] |
| | 5,802,226 individuals | CV events in NAFLD at fibrosis stage | 2·50° [1·68–3·72] |

such as age, gender, body mass index (BMI), waist circumference, smoking status, hypertension, or dyslipidemia [9,13–15]. Briefly, three meta-analyses including both diabetic and nondiabetic individuals indicated that NAFLD increases the relative risk of CVD, by 1.64 (95% CI: 1.26–2.13), 1.88 (95% CI: 1.68–2.01), and 1.81 (95% CI: 1.23–2.66), respectively [9,13,14]. Zhou et al. also explored the correlation between NAFLD and CVD among adults with diabetes [15]. Based on 11 observational studies, authors found that diabetic patients with NAFLD showed a two-fold increased risk for CVD compared with the non-NAFLD group concluding that NAFLD and type 2 diabetes (T2DM) might have a synergistic effect on CVD risk [15]. Several lines of evidence also linked NAFLD with subclinical markers of atherosclerosis such as increased carotid artery intima-media thickness (IMT), arterial stiffness, coronary calcification, endothelial dysfunction, and impaired left ventricular diastolic function [15]. In a second meta-analysis, Zhou et al. [16] found that NAFLD associated with increased IMT/plaques [OR 1.74; 95%CI (1.47–2.06)], increased arterial stiffness [OR 1.56; 95%CI (1.24–1.96)], coronary artery calcification [OR 1.40; 95%CI (1.22–1.60)] and endothelial cell dysfunction [OR 3.73; 95%CI (0.99–14.1)]. However, these results must be interpreted with caution due to the heterogeneity of the populations included in these meta-analyses and the observational nature of the studies, which fail to identify any causal or temporal relationships.

Indeed, despite the large amount of evidence linking NAFLD to ASCVD, there are several open issues on this topic. It is still debated whether this association derives from the overlap of common risk factors or aetiologies or if hepatic steatosis independently contributes to an increased atherosclerotic risk. Moreover, it is still highly unpredictable which NAFLD patient will develop ASCVD or severe liver complications. Interest is growing in identifying patients' characteristics able to predict the different lines of disease progression.

In the present review, we aim to provide evidence supporting the existence of different forms of NAFLD sub-phenotypes and the need to separate the metabolic from the genetic disease determinants to disentangle the intricate relationship between NAFLD and ASCVD.

## 2. NAFLD: a spectrum of pathophysiological conditions

NAFLD is a heterogeneous and complex disease, whose natural course may include cardiovascular, metabolic, neoplastic or liver-related complications. Both metabolic and genetic factors influence this heterogeneity [17]. The structural dissection of the differential pathways involved shall help us in identifying precise disease subtyping to predict individualised CVD risk.

### *2.1. The intricate relationship between NAFLD and cardiometabolic disorders*

The most common NAFLD phenotype is associated with metabolic dysfunction. Recently, a consensus of international experts in fatty liver disease have agreed that the NAFLD term does not reflect the knowledge on the heterogeneity of the pathophysiological mechanisms associated with the disease. Metabolic-associated fatty liver disease (MAFLD), has been suggested as a more proper term [18] to emphasise the role of metabolic alterations on the pathogenesis of fatty liver disease. Based on this consensus, the major metabolic associated disorders leading to MAFLD are obesity and/or diabetes and/or at least two out of five features of metabolic syndrome (MetS) [18].

The strong association between NAFLD and obesity was well demonstrated in the European DIONYSOS study cohort including 3000 participants. Notably, NAFLD was present in 25% of participants with a normal weight (BMI 20.0–24.9 kg/m$^2$), 67% of overweight participants (BMI 25.0–29.9 kg/m$^2$) and 94% of participants with obesity (BMI ~30 kg/m$^2$) [19]. The worldwide prevalence of obesity in NAFLD and NASH patients have been reported to be 51% and 81%, respectively [7]. Therefore, it is generally accepted that the initiating events in NAFLD are dependent on the development of obesity and insulin resistance [20,21].

Several studies consistently demonstrated a strong association between NAFLD and the features of metabolic syndrome (MetS) [22–25] so that NAFLD is currently considered as the liver feature of MetS pushing in naming it as metabolic fatty liver disease, MAFLD [23,24,26,27]. Roughly, 42% of NAFLD subjects have MetS, 69% hyperlipidemia, 39% hypertension and 22% diabetes [7]. High prevalence of NAFLD has been reported in patients with T2DM, ranging from 40% to 70% [28,29]. Conversely, the prevalence of NAFLD in patients with Type 1 diabetes mellitus is relatively low (8.8%) [30] thus further supporting the hypothesis that insulin resistance, which is typically present in obesity and T2DM, but rarely in T1DM, is the main contributor in the pathogenesis of MAFLD [31].

To this regard, it is worth to mention that the accumulation of visceral adipose tissue (VAT), frequently associated with hepatic steatosis, represents a strong determinant of complications usually seen in NAFLD patients such as atherogenic dyslipidaemia, insulin resistance, pro-inflammatory states, and elevated blood pressure [32]. The portal/fatty acid flux theory proposes that visceral fat, through its increased lipolytic activity, releases toxic free fatty acids (FFA), which are carried directly by portal circulation into the liver in high concentrations leading to the development of hepatic insulin resistance through the accumulation of hepatic fat [33]. During this process visceral fat also releases inflammatory cytokines, contributing to the inflammatory milieu that represents a potent driver of promoting atherosclerosis process [34,35]. At the same time, it is known that the serum concentrations of adipokines, derived from visceral fat as well as some proinflammatory cytokine in overweight patients, are closely linked with insulin resistance and NAFLD development [36].

Given the metabolic hallmark of the new definition of MAFLD, it could be speculated that an increased risk of cardiovascular complications should be expected in this subtype of NAFLD [37]. Indeed, despite data about cardiovascular abnormalities in MAFLD are limited, it has

been showed that individuals with MAFLD phenotype had a substantially increased ASCVD risk compared to those without MAFLD [38]. However, while the new MAFLD definition better outlines the syndromic of NAFLD and encloses the large majority of fatty liver patients, it excludes those with metabolic-healthy NAFLD, that could be probably considered as the "*pure*" genetic ones [39]. These patients might represent a useful human model to evaluate the potential individual association between fatty liver *per se* and ASCVD.

### 2.2. NAFLD genetics

Thanks to the recent discoveries on genetic factors predisposing to NAFLD, the opportunity to classify the disease has been enriched by the chance to cluster the 'genetic' sub-phenotypes of NAFLD.

An increasing number of single-nucleotide polymorphisms (SNPs) have been discovered as associated with the presence and severity of NAFLD, namely, *PNPLA3*, transmembrane 6 superfamily member 2 (*TM6SF2*), glucokinase regulator (*GCKR*), membrane bound *O*-acyltransferase domain-containing 7 (*MBOAT7*) [40–47]. In addition, the hydroxysteroid 17β-dehydrogenase (*HSD17B13*) and the rs2642438 (p. A165T) variant in the mitochondrial amidoxime reducing component 1 (*MARC1*) have been found to be associated with protection from NAFLD progression and all-cause cirrhosis, respectively [48–50]. Interestingly, most of these variants were found to have a plausible pathophysiological role in causing lipids alteration in hepatocytes, such as in droplets retention or very-low density lipoproteins (VLDLs) secretion, impaired synthesis or catabolism determining lipotoxicity [51].

Nowadays, the rs738409 polymorphism in *PNPLA3* (I148 M) is the most potent gene variant predicting NAFLD risk [45,46]. Interestingly, the variant associates with the full spectrum of NAFLD (from NASH to fibrosis, cirrhosis and HCC) [42,43,46,52,53], in absence of body weight alterations, insulin resistance or the MetS [54]. Overall, these findings shed light on the hypothesis that liver fat accumulation *per se* is not a risk factor for atherosclerosis.

Over the past years, in an attempt to separate patients in whom hepatic fat is due to metabolic alterations from those in whom it is due to genetic factors, we enrolled two groups of NAFLD patients. One group was carrying metabolic disorders and was wild-type for *PNPLA3* variant (MetS-related NAFLD) the other included metabolic healthy NAFLD patients carrying the pro-steatogenic rs738409 *PNPLA3* GG genotype [39]. The latter group represents a small proportion of NAFLD patients expressing a "*pure genetic NAFLD*", which should not be confused with lean NAFLD. It must be noted that 'lean NAFLD' definition has been often used to identify patients with low metabolic load to set against dysmetabolic ones. However, many lines of evidence have proved that these patients might have metabolic dysfunction (e.g.: insulin resistance, diabetes, hypertension [55]) and, based on these cardio-metabolic risk profiles, might be included in the new MAFLD definition [56]. Not by chance, lean NAFLD patients have similar risk to develop cardiometabolic and liver complications as compared to obese ones [57].

By identifying these two distinct forms of NAFLD, we demonstrated that the hepatic fat excess increased the burden of subclinical atherosclerosis, only when associated with metabolic disturbances [39]. Moreover, we also identified an impairment in HDL functionality, namely reduced HDL-mediated cholesterol efflux capacity (CEC), in metabolically- but not in genetically- driven NAFLD [58]. As altered HDL-mediated cholesterol efflux is associated with increased risk of ASCVD, these findings further suggest the existence of pro-atherogenic pathways which are differently active in metabolically- and genetically- driven NAFLD. Several Mendelian randomization studies have confirmed these findings. Lauridsen B.K. et al. [59] found that *PNPLA3* gene variant was not associated with increased risk of CHD in the general population, despite causing a high risk of the entire spectrum of NAFLD. Recently, Grimaudo et al. [43] in the first prospective study on *PNPLA3* gene variants carriers, found that this variant was independently associated with a higher risk of liver disease progression and liver-related death (HR, 3.64, 95% CI, 1.18–11.2; $p = 0.02$), but not with cardiovascular events [43]. Overall, these results confirm the notion that the subset of metabolically healthy patients carrying the rs738409 *PNPLA3* variant are potentially affected by genetic NAFLD without increased ASCVD risk [54].

Also the *TM6SF2* C > T (E167K) rs58542926 variant was found to predispose patients to hepatic fat accumulation [44]. This variant has been reported to interfere with the efflux of fat from hepatocytes, by reducing apolipoprotein B (apoB) lipidation and, thereby, VLDLs secretion [60]. Interestingly, several studies have demonstrated that NAFLD risk and CVD risk do not coincide in carriers of the *TM6SF2* 167K pro-steatogenic allele [60,61]. Thus, both carriers of the rs738409 G allele in *PNPLA3* and rs58592926 T allele in *TM6SF2* were found to be at higher risk of NAFLD hepatic progression rather than cardiometabolic complications (see Table 2).

Despite this, it must be noted that the "*genetic NAFLD sub-phenotype*" should not be considered as a fully separated entity. In fact, the genetic pro-steatogenic background interacts with the environment in generating its pathological effect. A previously published study highlighted the synergistic interaction between obesity and three NAFLD risk variants (*PNPLA3* I148 M, *TM6SF2 E167K* and *GCKR P446L*) across the entire spectrum of NAFLD [62]. Specifically, the steatogenic effect of the M-allele in *PNPLA3* was amplified by obesity (BMI ≥30 kg/m). A similar amplifying effect of BMI on hepatic triglycerides content (HTGC) was seen for the *TM6SF2* risk variant (E167K), with a less significant interaction ($p$-$_{\text{interaction}} = 0.006$), likely due to the lower frequency of the risk allele when compared to the *GCKR* risk allele. Thus, the interaction between genotypes and BMI on hepatic fat content is not unique to *PNPLA3* [62]. Probably, these "mixed" patients have higher cardiometabolic risk in comparison to those "purely" genetics (pure genetic NAFLD group). Thus, based on the current knowledge, we could identify at least three clinical subtypes of NAFLD (Fig. 1).

In conclusion, it could be reasonable to speculate that hepatic fat excess *per se* is unlikely to cause CVD and the reported epidemiological association is mostly mediated by the classical risk factors for atherosclerosis (dyslipidemia, diabetes mellitus, insulin resistance). However, the underlying mechanisms linking NAFLD to CVD are very complex and involve several different metabolic pathways (see below).

## 3. Mechanisms linking NAFLD to ASCVD

Causal mechanisms underlying the association between NAFLD, and CVD are not fully understood. However, several pathophysiological pathways including pro-atherogenic lipid alterations (e.g. atherogenic dyslipidaemia), insulin resistance, low-grade inflammation, *de novo* lipogenesis (DNL), endothelial dysfunction and oxidative stress and, more recently abnormal microbiota, have been described as links between NAFLD and ASCVD.

### 3.1. Lipids

As mentioned above, NAFLD is usually associated with MetS, insulin resistance, and T2DM, which are characterised by the presence of a typical lipid plasma alteration called atherogenic dyslipidemia (AD). AD is defined as the combination of elevated concentration of TG-rich lipoproteins (TRLs; ie, very low-density lipoproteins - VLDLs - plus chylomicrons and their remnants), lower concentration of high-density lipoprotein cholesterol (HDL-C), and an increased proportion of circulating small-dense particles of low density lipoprotein cholesterol (LDL-C) [63–65].

As TRLs contain apolipoprotein B (apoB), plasma concentration of apoB is typically elevated in NAFLD, thus representing an helpful biochemical marker for the overall lipoprotein alterations as well as CVD risk in this condition [66].

To this regard, it is worth to mention that the over secretion of apoB-TRLs particles together with their impaired clearance by the liver are the

**Table 2**
Eeffects of *PNPLA3* and *TM6SF2* gene variants on liver disease severity and CVD risk.

| *PNPLA3* | Cohort | Severity of liver diseases | | | | | Cardiometabolic risk |
|---|---|---|---|---|---|---|---|
| | | NAFLD | NASH | Fibrosis | Cirrhosis | HCC | |
| [54] | NAFLD | | | | | | |
| [129] | NAFLD | | 3.43 | | | | |
| [130] | NAFLD<br>NASH | 3.12 | 3.26 | 3.37 | | | ↓BMI, T2DM, TG, WC |
| [46] | NAFLD | 1.35 | 1.5 | 1.54 | | | |
| [45] meta-analysis | NAFLD | 3.26 | 3.26 | 3.25 | | | No association with BMI or HOMA-IR |
| [131] | Mixed cohort | | | | 1.86 | | |
| [132] | OBESITY | | | | | 7.42<br>HR:5.88 | |
| [133] | NAFLD | | | | | | ↑IMT |
| [52] | NAFLD-related HCC | | | | | 2.26 | |
| [134] meta-analysis | Mixed | | | 1.35 | 1.32 | 1.40 | |
| [135] meta-analysis | Mixed | | | | 1.86 | | |
| [136] meta-analysis | NAFLD | 1.92 | | | | | |
| [137] meta-analysis | NAFLD | 3.41 | 4.44 | 3.11 | | | |
| [60] | NAFLD NASH | | 1.65 | 2.08 | | | |
| [138] | CARDIoGRAMplusC4D cohort | | | | | | ↓CAD |
| [139] | NAFLD | 2.42 | | 1.67 | | | |
| [59] | Danish general population | 2.03 | | | 3.28 | | ↓IHD |
| [39] | NAFLD | | | | | | ↓IMT |
| [140] | HCC discovery cohort | | | | | 1.67 | |
| [43] | NAFLD or NAFLD-related cirrhosis | | | | HR:2.68 | | no CVD events |
| [141] | NAFLD | 1.53 | | | | | ↓CHD |
| ***TM6SF2*** | | **NAFLD** | **NASH** | **Fibrosis** | **Cirrhosis** | **HCC** | |
| [44] | NAFLD | | | | | | ↓LDL-C ↓TG |
| [61] | Population-based cohort in Norway | | | | | | ↓TC ↓TG<br>↓ MI |
| [52] | NAFLD | | | 2.94 | | | |
| [60] | NAFLD NASH | | 1.84 | 2.08 | | | ↓ TC ↓ TG<br>↓ Carotid plaques |
| [142] | NAFLD NASH | 1.37 | no | no | | | ↓ CVD risk |
| [139] | NAFLD | 4.72 | | 1.76 | | | |
| [138] | CARDIoGRAMplusC4D cohort | | | | | | ↓ CAD |
| [140] | HCC discovery cohort | | | | | 1.45 | |
| [143] | UK Biobank with MRS | | | | | | ↓CAD |

The magnitude of the effect is expressed by odd ratios (OR) values.
Studies exclusively focused on alcoholic fatty liver disease (ALD) or chronic hepatitis study populations were not considered. OR were extracted from those obtained by multivariate analysis in single articles and by overall Fixed term from meta-analysis.
BMI, body mass index, CAD, coronary artery disease, CHD, coronary heart disease, CVD, cardiovascular disease, HOMA-IR, homeostasis model assessment-insulin resistance, HR, Hazard ratio, IHD, ischemic heart disease, IMT, intima-media thickness, LDL-C, low-density lipoprotein cholesterol, MI, myocardial infarction, TG, triglycerides, T2DM, type 2 diabetes, WC, waist circumference.

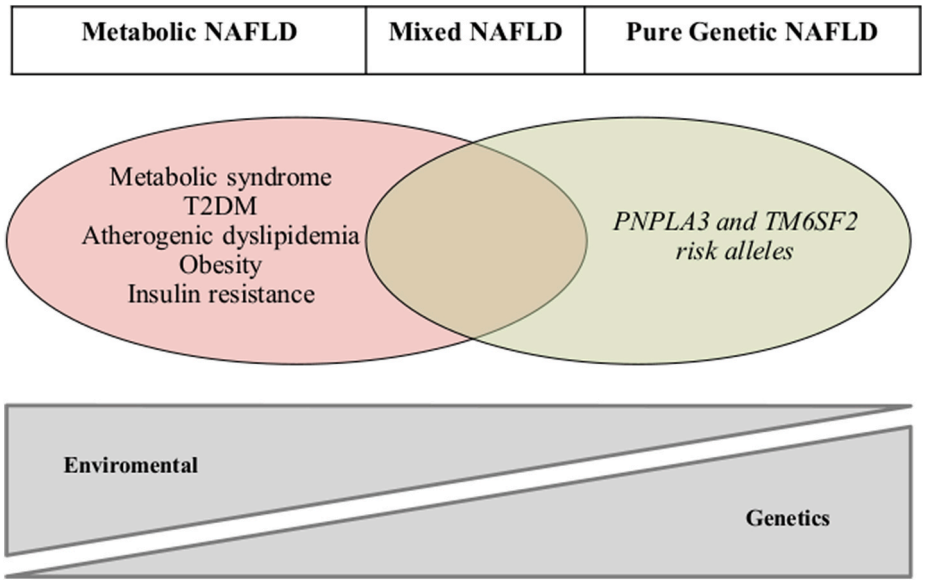


**Fig. 1.** NAFLD subphenotypes.

main causes of hepatic fat accumulation and central to the development of hyperlipidemia. Interestingly, this typical lipid profile might also depend on the reduced activity of lipoprotein lipase (LPL) due to an increased activity of ANGPTL3 and ANGPTL4, which represent the physiological circulating inhibitors of LPL [67–69]. Notably, plasma levels of ANGPTL3 and ANGPTL4 were found to be increased in obesity and T2DM as well as in NAFLD/NASH patients [70].

Elevated TGs are typically found in MAFLD [71]. Notably, higher TGs, remnant lipoprotein cholesterol (RLP-C) and high TRLs have been observationally and genetically associated with an increased risk of development of atherosclerosis [72,73], thus representing another potential pathophysiological link between NAFLD and ASCVD. Recently, in the post-hoc analysis of the Plinio study, higher levels of RLP-C were found in NAFLD patients in comparison to those without. In addition, among NAFLD patients, those with higher baseline RLP-C had a more than doubled risk of developing cardiovascular events during follow up [74]. On the other hand, AD was found to be associated with advanced liver fibrosis in diabetic NAFLD patients [75].

Altered levels of other apoB-containing lipoproteins, namely small-dense LDL, which are more atherogenic than normal-sized LDL, have been described in patients with NAFLD [66]. NAFLD patients may present low levels of larger LDL-1 and increased smaller LDL-3 and LDL-4 particles leading to a more atherogenic profile. Interestingly, in 2011, Athyros et al. [76] demonstrated that treating LDL intensely with atorvastatin reduced both markers of liver damage and cardiovascular events incidence more than a more cautious treatment, suggesting a role of LDL as marker of both liver and cardiovascular risk. Moreover, in the Greece study, atorvastatin treatment improved liver tests and reduced cardiovascular morbidity in patients with mild-to-moderately abnormal liver tests that were potentially attributable to NAFLD [77].

Another hallmark of MAFLD-associated dyslipidemia is the alteration of HDL profile. By evaluating HDL particle subfractions, Bril et al. [78] found smaller HDL particles among patients with NAFLD. Of note, it has been previously reported, that these small HDL particles have poor functionality, and that their clearance is increased, contributing to lower HDL cholesterol and apoAI seen in these patients [79]. It has been postulated that HDL exerts an important antioxidative function, among other antiatherogenic activities. Such antioxidative capacity can be crucial for the growth and complications of atherosclerotic plaques [80]. Finally, increased liver fat content was found to be closely correlated with a more prolonged postprandial lipemia as estimated by measurements of chylomicrons, VLDL1 TG, and apoB-48 increases after an oral fat load [81]. In addition, NAFLD was also associated with increased levels of oxidised LDL after an oral fat load [82]. Postprandial lipid levels contribute substantially to the hepatic TG content in NAFLD and have been reported to be an independent cardio metabolic risk factor [81,83,84].

### 3.2. Insulin resistance, glucose metabolism and de novo lipogenesis (DNL)

Abnormal glucose metabolism and insulin resistance are the major hallmarks of NAFLD. Both are crucial in NAFLD and CVD pathogenesis [85]. Disorders of glucose metabolism in NAFLD patients can be attributed to visceral obesity in conjunction with increased body weight which in turn is associated with insulin resistance.

Insulin resistance is accompanied by a persistent hyperinsulinemia, that is of central importance for the induction and maintenance of an unfavourable metabolic condition characterised by increased free fatty acids (FFA) and glucose levels, which subsequently promotes the development of cardiometabolic disorders. Insulin resistance is regarded as a major driving force for atherogenic dyslipidaemia because it increases FFA release, which stimulates hepatic triacylglycerol output [86].

Insulin resistance also drives hepatic *de novo* lipogenesis (DNL) in NAFLD [87]. DNL contribution to liver TG content is less than 5% in healthy control subjects but contributes to liver triglyceride content for 26% and to VLDL triglycerides for a total of 23% in hyperinsulinemic subjects with NAFLD [88]. Interestingly, in the work by Donelly KL et al., the DNL was increased in fasted and fed states, indicating that conceivably the elevation of transcription factors and enzymes are involved in hepatic DNL [88]. These include the transcription factor SREBP-1c, which is activated by insulin, and activates many important enzymes involved in lipogenesis. Carbohydrate response element–binding protein (ChREBP) also regulates DNL by activating several enzymes involved in the lipogenesis and pyruvate kinase, a key regulator of glycolysis [89]. Notably, the rs1260326 *GCKR* gene variant, encoding for the loss-of-function variant (P446L), results in a net increase in intracellular glucose phosphate, leading to increase DNL via the induction of glycolysis and the stimulation of the ChREBP [51]. The variant was found to be associated with higher susceptibility to NAFLD and advanced liver disease as described above [90].

Between NAFLD and diabetes, as already discussed above, there is a tight interplay and diabetes is one of the most important atherosclerotic risk factors in patients with NAFLD [91]. It is worth mentioning that some glucose-lowering drugs have shown potential in ameliorating NAFLD. Data from randomised control data have highlighted that pioglitazone seems to be of some help in non-cirrhotic, non-diabetic adults with biopsy-confirmed NASH. More recently, a novel class of drugs, the glucagon-like peptide-1 receptor agonist (GLP-1RA), have been found to be of potential interest in treating NAFLD in diabetic patients. A recent meta-analysis by Mantovani et al. [92] have showed that GLP-1RAs treatment was able to reduce the absolute percentage of liver fat content (as assessed by magnetic resonance-based techniques), as well as to induce histological resolution of NASH without worsening liver fibrosis. Nevertheless, the use of these drugs is actually a matter of debate. While several other studies have confirmed the potential use of thiazolidinediones (TZDs) for selected NAFLD patients, some others did not support findings regarding the beneficial effect of GLP-1RA [92]. Preliminary but equally promising results concern the sodium-glucose cotransporter-2 (SGTL-2). They markedly reduce the atherosclerotic risk [93] and seem to be able to reduce liver-related risk in NAFLD patients [94].

### 3.3. Endothelial cell dysfunction

In recent years, an increasing amount of evidence has shown that patients with NAFLD have endothelial dysfunction (ED) [95], a marker of early atherosclerosis [96]. Endothelial cells are a crucial component of the normal vascular wall. When the endothelium is injured, it loses its specialised properties, resulting in ED which is a hallmark of vascular diseases [97]. Activation and damage of the endothelial monolayer seem to trigger the development of the atherosclerotic lesions damaging. Once the integrity of endothelium is interrupted, lipid penetration and mononuclear cell adhesion might be initiated. Also, the expression of adhesion molecules (V-CAM in particular), the secretion of proinflammatory chemokines, the reduced production of nitric oxide and reduced endothelial-mediated vasodilation impairing the vascular response to physiologic and pharmacologic stimuli, have been described [98]. Atherosclerosis risk factors, commonly found in NAFLD patients, such as hyperlipidemia, hypertension, diabetes mellitus, smoking, and infections can directly or indirectly stress the arterial endothelium, resulting in its dysfunction, damage, or both [99]. Several pieces of evidence have shown that markers of endothelial dysfunction such as flow-mediated dilatation percentage (FMD%), are significantly impaired in patients with NAFLD [100,101] with a significant gradient according to liver disease severity [102]. In addition, FMD impairment was found to correlate with Framingham risk score in NAFLD patients [103], enforcing the hypothesis of the role of endothelial dysfunction in the increased cardiovascular risk in NAFLD. Also, the average of IMT, used as a non-invasively marker of endothelial dysfunction and arteriopathy, was significantly increased in NAFLD cases as compared with non NAFLD [104,105]. In a recent meta-analysis, NAFLD patients had more carotid atherosclerosis than controls, with enlarged mean IMT and higher plaque prevalence [106].

### 3.4. Oxidative stress

Oxidative stress is associated with many chronic diseases, especially those characterised by low-grade inflammation, such as diabetes, metabolic syndrome, and obesity. Excessive reactive oxygen species (ROS) production may induce Kupfer cell and stellate cell activation in the liver thus promoting fibrosis and NAFLD progression to NASH and cirrhosis. Oxidative stress is also an important factor for the pathogenesis and progression of cardiovascular disease. It has been suggested that it may be a possible mechanism linking NAFLD to cardiovascular disease [107,108]. Oxidative stress and the accumulation of reactive oxygen species in plasma favours endothelial inflammation.

Increased oxidative stress was documented in vivo in a large cohort of subjects with NAFLD by measuring the urinary levels of 8-iso-prostaglandin F2α (8-iso-PGF2α), which results from the non-enzymatic oxidation of arachidonic acid. This was measured together with the serum levels of the soluble Nox2-derived peptide (sNox2-dp), which represents an indicator of Nox2 activation, the main isoform of NADPH-oxidase responsible for ROS production [109]. Plasma accumulation of ROS, proportional to NAFLD/NASH severity, triggers endothelial inflammation and ultimately atherosclerosis progression [110]. In addition, oxidative stress may be the consequence of the reduction of circulating levels of antioxidant systems. To this regard, in NAFLD patients, independently from the severity of liver disease, we observed a reduction of circulating levels of Vitamin E [111].

### 3.5. Gut microbiota

Gut microbiota influences different metabolic pathways, and its alterations are widely recognized as a significant pathophysiological mechanism in metabolic disorders onset [112]. In addition to obesity and T2DM, an association between microbiota dysregulation (i.e. dysbiosis) and cardiovascular disease was found [112]. Patients developing cardiovascular disease show less abundance of *Bacteroides, Alistipes* and *Oscillibacter spp.* and of *Faecalibacterium prausnitzii* while show more abundance of *Ruminococcus, Acinetobacter, Escherichia coli, Enterobacter aerogenes, Klebsiella spp, Veillonella spp* [113–115]. A recent meta-analysis [116] summarised findings on gut microbiota composition in NAFLD, reporting the increase of *Streptococcus, Prevotella* and *Escherichia* genera, and the decrease of *Faecalibacterium, Coprococcus and Ruminococcus* genera. Authors suggested that changes of *Faecalibacterium* and *Prevotella* abundance were associated with BMI, while the change of *Streptococcus* and *Faecalibacterium* quantity were associated with inflammatory status. Some of these results overlap with those observed in patients at high risk for CVD (e.g changes in *Escherichia coli* abundance) thus partially explaining the link between NAFLD and CVD. In patients with atherosclerosis, gut microbiota produces high levels of trimethylamine (TMA) from dietary L-carnitine, phosphatidylcholine and lecithin. TMA is oxidised in trimethylamine N-oxide (TMAO) in the liver [117]. Notably, patients with high plasma levels of TMAO show increased risk for major cardiovascular events and all-cause mortality [118]. The link between TMAO and cardiovascular disease is due to the association between TMAO, increased oxidative stress [119], platelet activation [120] and lipid macrophage uptake, leading to foam cells formation [117]. Recent studies demonstrated that circulating levels of TMAO are associated with NASH diagnosis and circulating levels of bile acids [121] probably via the inhibition of hepatic farnesoid X receptor (FXR) activation [122]. Another mechanism by which gut microbiota dysregulation affects cardiovascular system health is represented by the lipopolysaccharides (LPS) translocation in blood stream [119]. LPS is a component of the bacterial lipid membrane in Gram-negative and a potent stimulator of innate immunity. LPS activates toll-like receptors (TLRs), in particular on the endothelial surface, leading to the inflammatory response and endothelial dysfunction [123] and on platelets surface inducing oxidative stress and platelet activation [124].

Finally, it was recently proved that LPS from *Escherichia coli* localises in human plaque and may contribute to atherosclerotic damage via TLR4-induced oxidative stress [125]. Notably, patients with NAFLD showed an increase of 38–40% of their LPS serum levels in comparison to dysmetabolic patients without NAFLD [108]. This increase in circulating LPS may be the consequence of several factors. The overgrowth of intestinal Gram-negative bacteria and the increased intestinal permeability have been found [108]. LPS localises in NASH patients' liver, particularly in hepatocytes and induces nuclear factor-κB activation [126]. In addition, LPS may promote macrophages-platelets (TLR4+) hepatic colocalization, suggesting a LPS-induced platelet activation in the liver [126].

## 4. Conclusions and future perspectives

Prevalence of NAFLD suggests that it is the liver disease of our century. Since patients with NAFLD present an increased CVD incidence, to predict what proportion of subjects will develop cardiovascular complications represents a public health issue.

To date, differently from other clinical conditions [127], a cardiovascular risk score specifically validated in NAFLD patients is missing. Identifying a tool for this purpose could represent one of the major challenges for clinical research on NAFLD and could address one of the unmet needs in the clinical management of these patients. Meanwhile, waiting to know if the incoming drugs resolving NAFLD will also reduce the cardiovascular risk of these patients, by treating cardiometabolic risk factors in those with NAFLD, we should consider fatty liver as a "compelling indication" in choosing the best drugs.

In the future, the recognition of the heterogeneous drivers responsible for the different disease subtypes of NAFLD, shall improve the development of integrated risk model of NAFLD – CVD progression based on genetic, molecular, histology and "omics" based data (transcriptome, metabolite, proteome, microbiome) toward a precision medicine approaches [128]. This would help in improving the design of clinical trials by pooling patients' subgroups with specific phenotypes and in applying customised therapeutic strategies able to prevent CV events occurrence.

## Author contributions

A.D.C, F.B and L.D researched data for the article, contributed substantially to discussion of the content, wrote the article, and reviewed and/or edited the manuscript before submission. S.B, D.P, F.A, M.D.B and M.A contributed substantially to discussion of the content and reviewed and/or edited the manuscript before submission.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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