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Review article

Does nonalcoholic fatty liver disease cause cardiovascular disease? Current knowledge and gaps

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HIGHLIGHTS

- NAFLD is highly prevalent ranging from steatosis to cirrhosis.
- NAFLD advanced forms associated with cardiovascular risk.
- NAFLD studies limited by heterogeneity and confounders.
- Genetic variants predisposing to NAFLD not consistently associated with cardiovascular events.
- No proof that NAFLD is causal of cardiovascular disease.

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is highly prevalent and includes a spectrum of abnormalities ranging from steatosis to cirrhosis. In this review, we address recent evidence and limitations of studies that evaluated the association of NAFLD with atherosclerotic cardiovascular disease. NAFLD is considered an ectopic fat deposit associated with metabolic (insulin resistance, hyperglycemia and dyslipidemia), inflammatory, coagulation and blood pressure disturbances. Prospective studies have associated NAFLD presence and severity, particularly steatohepatitis and fibrosis, with an increased risk of cardiovascular disease. However, these studies are limited by heterogeneity concerning NAFLD diagnostic criteria and disease severity stratification, as well as by the presence of confounding factors. In addition, genetic variants predisposing to NAFLD, such as the *PNPLA3* I148M mutation, were not consistently associated with an increased risk of cardiovascular events. Therefore, currently, it is not possible to prove a causal relation between NAFLD and cardiovascular disease. Furthermore, there is presently no evidence that NAFLD diagnosis can be used as a tool to improve cardiovascular risk stratification and modify treatment. Specific treatments for NAFLD are being developed and must be tested prospectively in adequately designed trials to determine the potential of reducing both hepatic and cardiovascular diseases and to prove whether NAFLD is indeed a cause of atherosclerosis.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) affects from 25 to 45% of the general adult population and up to 70% of type 2 diabetic individuals in Europe and North America [1]. NAFLD comprises a spectrum of conditions ranging from steatosis, non-alcoholic steatohepatitis (NASH), to liver fibrosis that may cause cirrhosis and hepatocellular carcinoma [2–6]. With the exception of few well-defined genetically determined forms [7], liver fat accumulation in NAFLD is part of a more extensive adipose excess co-existing with metabolic syndrome components, namely insulin resistance (IR), elevation in blood glucose and blood pressure, atherogenic dyslipidemia, and a low-grade systemic inflammatory state [2]. Indeed, a recent report from the US Third National Health and Nutrition Examination Survey (NHANES III) [8] shows that subjects with metabolic syndrome present an extremely high risk of NAFLD (adjusted odds ratio of 11.5, 95% confidence interval- CI 8.9–14.7). Despite this intrinsic association, epidemiological studies suggest an independent connection of NAFLD, and possibly its severity,

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with a greater risk of cardiovascular disease (CVD) development [3]. This association might be even more important if we consider the ongoing epidemics of obesity affecting adults, children and adolescents. According to a recent report, 30% and 6.5% of children with NAFLD diagnosis present with dysglycemia and type 2 diabetes (T2D), respectively [9].

NAFLD as an additional depot of visceral fat may exacerbate pathological mechanisms associated with excess of abdominal fat [10]. However, due to (a) limitations of classical epidemiological studies i.e. the concomitance of NAFLD with abdominal fat, the metabolic syndrome and T2D, (b) the absence of interventional studies aiming at reducing hepatic fat accumulation, inflammation and fibrosis, there is no conclusive evidence that it has an independent causal role in atherosclerotic CVD. Indeed, recent evidence shows the presence of genetic variants predisposing to NAFLD are not associated with a greater risk of CVD in the absence of metabolic syndrome components [11–13]. This latter finding suggests that the risk factors for NAFLD rather than liver fat accumulation *per se* might promote atherosclerosis development.

One important practical and contentious issue for health care providers is whether NAFLD diagnosis changes clinical management of individuals with the metabolic syndrome or T2D[14–16] when CVD prevention is concerned [2–14].

This literature review article focuses on the recent advances in understanding the possible mechanisms linking NAFLD to atherosclerosis and thrombosis and discusses controversies of its association with CVD risk. We illustrate the role of NAFLD genetic factors as instruments to estimate the causal effect of NAFLD on CVD. We review the available evidence of a possible utility of NAFLD detection for the refinement of CVD risk stratification beyond concomitant classical risk factors. Finally, we discuss current and future treatments that could impact NAFLD and prevent cardiovascular disease.

2. Methods

2.1. Search strategy and selection criteria

Searches of PubMed were performed by the 3 authors using the terms “atherosclerosis”, “cardiovascular disease”, “coronary heart disease”, “CHD”, “coronary artery disease”, “CAD”, “stroke”, “cardiovascular risk”, “blood glucose”, “diabetes”, “dyslipidemia”, “blood pressure”, “biomarkers”, “genetic epidemiological studies”, “genetic”, “insulin resistance”, “liver enzymes”, “NAFLD”, “non-alcoholic fatty liver”, “NAFL”, NAFLD”, “liver/hepatic steatosis”, “non-alcoholic steatohepatitis”, “NASH”, “fatty liver”, “obesity”, “statin”, “ezetimibe”. Considering the recent explosion of literature in the field and, in particular, of recent reviews and meta-analysis on the topic, we have limited in most cases the search timeframe to 2 and 5 years (up to October 2018), respectively, for review papers/meta-analyses and original papers, and considered the relevant literature cited in these papers. Only articles published in English were considered.

3. Criteria and biomarkers for NAFLD diagnosis, severity, and their limitations

Table 1 shows diagnostic criteria as well limitations of available biomarkers for NAFLD diagnosis and staging. NAFLD is a progressive disease ranging from steatosis to NASH, liver fibrosis and chirrosis [2–5,17]. In clinical practice, NAFLD is suspected in overweight/obese individuals with the metabolic syndrome and/or T2D and usually diagnosed by hepatic ultrasound in the absence of other causes of liver disease, like excessive alcohol consumption, hepatitis or iron overload [18]. There is evidence that 1 in every 4 individuals with steatosis may progress to NASH [5]. Prospective long term follow-up histological studies have shown that NASH, especially when advanced forms of liver fibrosis (≥F2 fibrosis degree) are present [5], is associated with increased relative risk of liver and CVD mortality [19,20]. The gold standard for NASH and fibrosis diagnoses is liver biopsy, however, its widespread use is limited by its invasiveness, cost and adverse events [17]. Unfortunately, there is poor correlation between elevations in aminotransferases and severity of abnormalities in liver histology, and other diagnostic biomarkers for NASH and fibrosis are needed.

Table 1 summarizes imaging and metabolic biomarkers for disease staging. Research has focused on imaging biomarkers of fat as well as liver fibrosis, like ultrasound elastography or magnetic resonance imaging or spectroscopy [17]. In addition, serum metabolism biomarkers like blood cytokeratin-18 and biochemical panels including hyaluronic acid have been tested to diagnose more advanced stages of NAFLD, respectively NASH and fibrosis. These new biomarkers, however, suffer from limitations related to accuracy, costs and availability and liver biopsy is still the gold standard to diagnose and stage NAFLD.

4. New insights into the link between hepatic fat and cardiovascular disease: adiposity and insulin resistance, inflammation, lipoproteins, and coagulation

Fig. 1 summarizes the mechanisms associating excess caloric intake, NALFD and CVD. The most common cause of NAFLD is represented by excessive caloric intake coupled with reduced energy expenditure, due to low physical activity and reduced muscle fitness. In addition, there appears to be contributing independent deleterious effects of some nutrients like fructose [21], and saturated fat [22] on NAFLD development. Overall, these conditions lead initially to an expansion of the adipose tissue mass, low-grade inflammation and insulin resistance. In adipocytes, insulin resistance increases the rate of lipolysis, with consequent spillover of non-esterified fatty acids (NEFA) into ectopic depots: in the liver, skeletal muscle, and pancreas [12,23]. At the same time, compensatory hyperinsulinemia, which develops to maintain normal glucose homeostasis, drives *de novo* lipogenesis through the upregulation of the transcription factor Sterol Regulatory Element-Binding Protein-1c (SREBP-1c) [24].

NAFLD develops when the rate of hepatic synthesis of triglycerides exceeds NEFAs catabolism due to mitochondrial oxidation and their export as triglycerides in very-low density lipoproteins (VLDL) [10]. Therefore, NAFLD may be viewed as a consequence and therefore a good marker of insulin resistance severity. Indeed, several prospective studies have detected a robust association between NAFLD and risk of T2D development, as recently reviewed [12]. Within the context of systemic insulin resistance determined by environmental triggers [7,10], common genetic risk factors predispose to more severe hepatic fat accumulation. The mechanism underlying NAFLD consists of at least four different components as elucidated by human genetics: (a) impairment of intracellular triglyceride remodeling as for the *PNPLA3* gene variants, (b) VLDL secretion as for *TM6SF2* (transmembrane 6 superfamily member 2) variants, (c) impairment of phospholipid remodeling as for the *MBOAT7* (membrane bound O-acyltransferase domain containing 7) [25] variant and (d) increase in lipogenesis as for *GCKR* (glucokinase regulator) gene variant [7]. In carriers of all these gene variants, hepatic fat content is higher and these individuals have higher risk to have a progressive liver disease.

On the other hand, the liver plays a key role in glucose metabolism regulation and lipoprotein metabolism, and it has been surmised that hepatic fat accumulation *per se* promotes T2D by increasing hepatic insulin resistance [10]. However, given the strong epidemiological association and presence of unmeasured confounders in clinical studies, it has been so far very difficult to disentangle the relative contribution of hepatic *versus* visceral fat in the pathogenesis of insulin resistance and metabolic syndrome traits [26,27].

A body of evidence from experimental models indicates that hepatic *de novo* lipogenesis and glucose production are regulated by different pathways [12]. Omental and mesenteric adipocytes are more insulin-resistant and they release high levels of NEFA and cytokines into the

Table 1
Diagnostic criteria for different NAFLD stages, diagnostic methods/biomarkers and their limitations [5,6,17,18].

	Clinical features	Histological definition	Diagnostic methods/limitations
Steatosis	Usually overweight and obese individuals in the absence of other causes of liver disease like excess alcohol consumption, hepatitis and iron overload	At least 5% liver fat accumulation with no evidence of hepatocellular injury	Hepatic ultrasound (widely available, very specific, but poor sensitivity for < 12% fat accumulation); liver computed tomography (widely available, good sensitivity, but implicates in radiation exposure), magnetic resonance imaging-proton density fat fraction-PDFF (limited by costs) and ^{1}H-magnetic resonance spectroscopy (highly sensitive for small amount fat accumulation but not widespread available)
Steatohepatitis (NASH)	Overweight and obese individuals + features of the metabolic syndrome + elevated aminotransferases, where other causes of liver disease were not encountered	Steatosis associated with liver inflammation and hepatocyte injury (ballooning) independent of fibrosis stage	Liver biopsy gold standard (limited by invasiveness, risk of complications and high cost); elevated aminotransferases have low sensitivity and specificity for NASH; Blood cytokeratin-18 (CK-18) fragments predictors of NASH (limited by non-commercial availability and poor reproducibility); NASH test (includes age, BMI, blood biomarkers like alpha-2 microgobulin) and NASH diagnostics panel (including CK-18 fragments) not so accurate
Fibrosis	Individuals older age 45, and type 2 diabetics have a greater risk of fibrosis	Presence of fibrosis (scarring): F1-portal fibrosis not affecting septa; F2-portal fibrosis with few septa; F3-bridging septa between central and portal veins; F4-cirrhosis	Non-invasive detection of liver stiffness (elasticity) by elastography: ultrasound transient elastography (limited in severe obese) and magnetic resonance elastography are preferred tests over blood biomarkers; "simple" blood biomarkers (ALT. AST, platelet counts), markers of hepatic dysfunction, derived Fibrosis–IV score may be useful but sensitivity declines with age; elevated ferritin in the absence of hemochromatosis might indicate a greater risk of cirrhosis; markers of elevated collagen turnover (e.g. hyaluronic acid, aminoterminal peptide of pro-collagen III) still under investigation

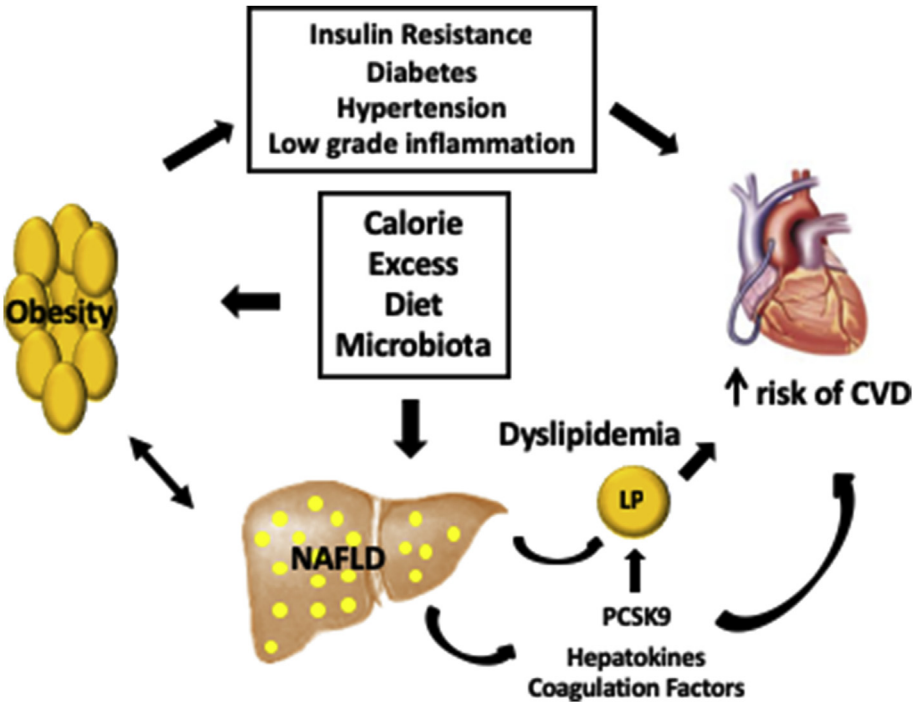

Fig. 1. Mechanisms of cardiovascular disease susceptibility due to excess of calorie intake.
A positive energy balance due to calorie excess or reduction in expenditure increases fat depot in the adipose and hepatic tissue. The fat in the adipose results in obesity, insulin resistance, T2D, hypertension and low-grade inflammation directly increasing the risk of cardiovascular events. The fat in the liver results in NAFLD, with consequent increased triglyceride-rich lipoproteins secretion causing dyslipidemia and increased risk of cardiovascular disease. NAFLD results also in changes in secretion of hepatic specific proteins including hepatokines, proprotein convertase subtilisin/kexin type 9 (PCSK9), coagulation factors inducing additional independent risk factors of cardiovascular disease. LP-lipoproteins (VLDL); ASCVD-atherosclerotic cardiovascular disease; NAFLD-non-alcoholic fatty liver disease.

portal vein [28], thus contributing to both systemic and hepatic inflammation. Reduced secretion of adiponectin, the main adipokine with insulin-sensitizing, anti-inflammatory, and anti-fibrotic effect [29], is also likely involved in the interplay between the liver and adipose tissue in NAFLD patients. Indeed, circulating adiponectin decreases with NAFLD severity [30], insulin resistance and adipose tissue inflammation, while experimental evidence suggests a protective role of this adipokine on hepatic fat and liver disease progression [31].

On the other hand, the recent observation that having high hepatic fat with low visceral fat is more deleterious than having high visceral fat with low hepatic fat [32] suggests a role of hepatic fat in the pathogenesis of IR. Increased amounts of NEFA are associated with lipotoxicity, activation of inflammatory pathways and the release of cytokines [33]. Accumulation of specific lipid species in hepatocytes may

Table 2
Impact of PNPLA3 I148M mutation* (rs738409) on cardiovascular disease and events.

Study	Design	N =	Ethnicity	Outcome	Main results
Petta et al. [52]	Cross-sectional cohort and prospective	429 patients with NAFLD	Europeans	cIMT	* Increased IMT in patients younger than 50 yrs (aOR 2.9, 1.1–7.7) * Faster cIMT progression
Posadas-Sanchez et al. [54]	Case-control study	2572 pCAD 1469 healthy	Hispanics	pCAD	* Increased risk of pCAD in T2D only (aOR 1.2, 1.01–1.42) * Increased insulin resistance and coronary calcifications in controls
Xu et al. [55]	Mendelian randomization in cohort study	19,925	Asians	IHD	* Reduced IHD (aOR 0.92, 0.87–0.97), but reduced serum lipids (pleiotropy)
Simons et al. [11]	Cohort studies	60,801 CAD 123,504 healthy	Mixed	CAD	* Borderline protection from CAD (unadjusted OR 0.96, 0.76–1.04)
Lauridsen et al. [13] Meta-analysis of Copenhagen studies and Copenhagen Studies with publicly available data from the CARDIoGRAMplusC4D consortium	Mendelian randomization prospective cohort study	From a total of 94,708, 10,897 with IHD 279,013 of whom 71,698 with IHD	Danish	IHD	OR: 0.95 (0.86–1.04) for IHD ($p = 0.46$) per M allele ORs for IHD per M-allele were 0.98 (0.96–1.00) using a fixed-effects model ($I2 = 0\%$; $p = 0.79$) and 0.98 (0.96–1.00) using a random-effects model

PNPLA3 I148M represents an ideal genetic variant to examine the impact of NAFLD on cardiovascular disease because it is a major determinant of hepatic fat content and disease with no major effects on the classical cardiovascular risk factors (circulating cholesterol, glucose levels, blood pressure).
CAD: coronary artery disease; pCAD: premature coronary artery disease; IHD: ischemic heart disease; cIMT: common carotid arteries intima-media thickness; OR: odds ratio, aOR: adjusted OR.

play an important role. For example, diacylglycerol interferes with insulin signaling by activating alternative Protein Kinase C (PKC) isoforms [23] in animal models, but this remains controversial in humans [34]. Recently, association studies and experimental data showed C:16-ceramides [35,36] and possibly lyso-phosphatidyl-cholines [37,38] as lipid mediators of hepatic insulin resistance in NAFLD.

Finally, the inflammatory state of NAFLD, namely NASH, may directly contribute to the systemic low-grade inflammation and to cardiometabolic disease through the production of various proteins secreted by the liver (e.g., interleukin-6, C-reactive protein, fibrinogen, monocyte chemotactic protein 1, and tumor necrosis factor-α, betatrophin, Fetuin-A) [33,39].

Intuitively, the composition of lipids stored in the liver may also affect lipoprotein composition and therefore contribute to atherosclerosis. Indeed, NAFLD severity is associated with an atherogenic lipoprotein profile mediated at least partially by insulin resistance [40]. Recent data also suggest association of severity of hepatic fat accumulation with circulating levels of PCSK9 (proprotein convertase subtilisin kexin type 9), a liver-derived peptide causing increased cardiovascular risk by inhibiting LDL uptake by hepatocytes [41].

NASH has also been linked with pro-thrombotic changes as shown by pro-coagulant imbalance and endogenous thrombin potential driven by an increased Factor VIII to Protein C ratio [42]. However, this association might depend on obesity and IR rather than on hepatic fat, as it is not found in patients who develop NAFLD due to strong genetic risk factors rather than IR [43,44]. The concurrent evaluation of genetic background will be important to dissect the possible causal role of hepatic fat on metabolic syndrome traits and cardiovascular disease in future studies.

Until recently, genetic studies did not provide evidence of a causal role of genetic variation determining NAFLD in the pathogenesis of IR and T2D[12]. On the other hand, the TM6SF2 E167K variant has been shown to decrease the risk of cardiovascular events since it decreases secretion of lipoproteins from the hepatocytes [45–47]. Conversely, *GCKR* gene variant increases circulating lipoproteins by lowering systemic glucose levels that is used as substrate to fuel *de novo* lipogenesis in the liver [48].

Recently, however, a Mendelian randomization study, using genetic variants in the *PNPLA3*, *TM6SF2*, *MBOAT7* and *GCKR* genes as instruments, has been carried out in three cohorts: a large collection of individuals with available liver biopsy (the Liver Biopsy Cohort), in a population-based sample study with measurement of liver fat by MRS (the Dallas Heart Study), and in a large cohort of obese individuals (the Swedish Obese Subject Study). This study suggests a causal relation between genetically determined fat accumulation that is independent of overall obesity and (a) liver fibrosis and damage, and (b) insulin resistance that seems to be mediated by severity of liver damage [49]. Indeed, in the same study, data from the UK biobank cohort show a modest association between these steatogenic variants and T2D, in line with recent data in large genetically characterized cohorts [50].

5. Genetics of NAFLD and CVD risk

NAFLD is often associated with the main risk factors for atherosclerosis and CVD (dyslipidemia, T2D and hypertension) [8]. As example, 41% of those with NAFLD have impaired glucose tolerance or T2D, and 35% have elevated triglyceride levels [4]. Because NAFLD coexists often with these risk factors, epidemiological studies cannot infer whether liver fat *per se* causes CVD.

Naturally occurring mutations in genes affecting directly liver fat content give insights into the nature of the association between NAFLD and CVD [11,47]. In principle, if a genetic variant predisposes to liver fat increase independently of other CVD risk factors, it should also increase the risk of CVD if liver fat content is directly causing CVD. In fact, this seems not to be the case. In the following paragraphs, we focus on the two of the most well replicated genetic mutations increasing

liver fat content and their effect on cardiovascular disease.

The PNPLA3 I148M variant is a mutation with high frequency in several ethnic groups [51,52]. This mutation is the most robust gene variant increasing liver fat and disease progression [7] by interfering with lipid metabolism in hepatocyte and hepatic stellate cells. The mutation causes an intracellular impairment in lipid droplet remodeling and VLDL secretion in hepatocyte, causing an increase in liver fat content [53]. If liver fat *per se* or liver inflammation/fibrosis was an independent cause of CVD, the prediction would be that the allele increasing liver fat would also increase the risk of CVD. The PNPLA3 I148M mutation is the ideal genetic factor to test this model, because it is the major genetic determinant of liver fat.

Independent studies (Table 2) found that the I148M mutation was associated with increased vascular damage in high risk Hispanic individuals with NAFLD [54], but showed no or even protective effect on CVD in Asians [55]. However, it should be noted that the I148M variant has possible pleiotropic effects that should be considered when interpreting the results, as it is associated with increased risk of T2D, but reduced lipid secretion and circulating lipid levels [49,50].

The TM6SF2 E167K variant increases liver fat content [45], inflammation and fibrosis [47] by interfering with the efflux of fat from the hepatocyte (i.e: lipidation and secretion of apolipoprotein B during VLDL secretion). As a result, individuals carrying the mutations have higher liver fat content, lower circulating levels of apolipoprotein B-rich lipoproteins (triglycerides and LDL-C) and, not surprisingly, a lower CVD risk [47]. However, due to the strong direct impact of the mutation on circulating LDL-C, this mutation cannot be used to infer the causal effect of steatosis on CVD.

Recently, a Mendelian randomization study aiming at dissecting the epidemiological association between NAFLD and cardiovascular disease has been carried out in two large European cohorts [13]. In this study, no evidence for a causal relationship was found between NAFLD and cardiovascular disease by examining genetic variants in two steatogenic variants in the *PNPLA3* and *TM6SF2* genes. Findings persisted when meta-analysed together with the CARDIoGRAMplus C4D consortium data.

Taken together, to date human genetics has not provided support to the hypothesis that primary accumulation of liver fat does cause cardiovascular disease. It may be therefore reasonable to speculate that the association observed between NAFLD and CVD is mostly mediated by the classical risk factors for atherosclerosis (dyslipidemia, diabetes mellitus, insulin resistance) and unmeasured confounders. However, before definite conclusions can be drawn, more data will have to be examined considering a larger number of genetic risk factors, which predispose to NAFLD without impairing hepatic lipid secretion.

6. Epidemiology of NALFD and CVD risk

Table 3 summarizes the controversies of epidemiological studies associating NAFLD with CVD risk. Recently, a study level meta-analysis evaluated the association of NAFLD with CVD [3]. Ten observational prospective and six retrospective studies comprising 34,043 individuals (36.3% with NAFLD diagnosed by radiological methods or histology) were included. A total of approximately 2600 cardiovascular events occurred (more than 70% CVD deaths) over a median follow up of 6.9 years. Individuals with NAFLD had a higher, adjusted for CVD risk factors, odds ratio [OR] for cardiovascular events (1.64, 95% CI 1.26–2.13, $I^2 = 83\%$) than those without NAFLD. Those with more severe NAFLD had a higher risk of developing fatal and non-fatal CVD events (OR 2.58; 1.78–3.75, $I^2 = 39\%$). Despite the rigorous methodology used by the authors, this meta-analysis is limited by (a) the high heterogeneity among the available studies including adjustment for different CVD risk factors, (b) the combination of prospective and retrospective observations, (c) an elevated chance of selection bias in some studies and (d) the absence of individual data.

Overall, epidemiological studies cannot infer a causal relationship between NAFLD and CVD. Indeed, even considering the use of robust statistical methods, NAFLD cannot be dissociated from established risk factors for atherosclerosis. Moreover, other confounding factors, such as diet, physical activity, body composition, microbiota and environmental exposures, have not been examined in the available studies. Mendelian randomization studies, using genetic variants primarily increasing liver fat, with an elevated number of study subjects with lifelong exposure, in the absence of confounders encountered in classical epidemiological studies, have been useful to clarify this issue [14] (see previous section).

6.1. Does NAFLD presence change CVD risk assessment?

One important clinical practice issue is if NAFLD diagnosis confers an additional CVD risk when concomitant atherosclerotic risk factors are already considered. In clinical practice, to be used as a tool to stratify CVD risk, NAFLD diagnosis should comply with least three criteria [56]: (a) NAFLD diagnosis must show an independent higher relative risk of CVD events after adjustment for established risk factors; (b) it needs to improve risk discrimination when included in models comprising these established risk factors and (c) most importantly, NAFLD diagnosis needs to show the potential to reclassify CVD risk, i.e. to correctly move a patient to a higher CVD risk degree, warranting a more aggressive therapeutic approach. Unfortunately, there is scant and controversial evidence on the value of NAFLD presence and severity as markers for CVD risk stratification over and above classical risk factors [14–16] (Table 3). This issue is aggravated by the lack of consensus on how to quantify and qualify NAFLD severity e.g. different imaging methods, liver enzymes, biochemical scores or biopsy.

One study suggests that the use of classical risk factors would be adequate to evaluate CVD risk in NAFLD individuals. Treeprasertsuk et al. [57] showed that the Framingham risk score had a good accuracy in identifying coronary heart disease risk in a prospective US study cohort with 309 NAFLD patients diagnosed by liver imaging or biopsy (age 49.1 ± 11.1 years, 95% caucasian and 47% men) and followed by a mean of 11.5 years. In this study, risk factors for a first coronary event were the same in those with and without NAFLD, suggesting the absence of a specific mechanism induced by the latter in causing CVD. However, the study was limited by the relatively small sample size and the absence of an independent replication cohort.

The association between baseline serum alanine aminotransferase (ALT) levels, a surrogate marker of NALFD severity [1], and cardiovascular disease onset was evalauted in a cohort of 2430 Iranian T2D individuals followed for 20 years [14]. An inverse association between ALT levels > 30 IU/L and CVD in fully adjusted models (HR = 0.204, 95%CI 0.060–0.689) was detected. Of interest, the addition of ALT to Framingham risk scores calibrated for the Iranian population increased risk discrimination (area under the ROC curves from 0.728 to 0.733), and allowed risk reclassification in 9% of the studied subjects. However, those with higher AST levels were actually reclassified to a lower risk level.

Similarly, the Dutch Prevention of Renal and Vascular End-stage Disease (PREVEND) study showed an inverse association of ALT and AST serum enzymatic activities with CVD risk among 6899 subjects after a median follow-up of 10.5 years [15]. For one standard deviation of increment in ALT and AST concentrations, a 0.88 hazard ratio HR (95% CI 0.80–0.96) and 0.92 HR (0.84–0.99) for CVD onset were detected, respectively. However, in this study the addition of liver enzymes to classical risk factors for atherosclerosis neither changed CVD risk discrimination nor risk reclassification.

Simon et al. [58]. have found in a *post-hoc* analysis of IMPROVE-IT (Improved Reduction of Outcomes: Vytorin Efficacy International Trial) that the nonalcoholic fatty liver score (NFS), a marker of NAFLD severity that includes age, body mass index, AST, ALT, platelets, albumin and diabetes, was an independent predictor of recurrent cardiovascular events. The score also indicated individuals that would have a greater

Table 3
Characteristics and limitations of recent studies showing an association between NAFLD and increased cardiovascular risk and its role on risk stratification.

Type of study	Results and strengths	Main limitations
NAFLD and association with CVD risk		
Study level meta-analysis [3] including 34,043 subjects, 36% with NAFLD diagnosed by imaging or histology, median follow-up 6.9 years and 2600 cardiovascular events (> 70% deaths)	NAFLD associated independently with CVD (OR 1.64; 95%CI 1.26–2.13). More severe liver disease associated with a greater CVD risk (OR 2.58; 1.78–3.75, I^2 = 39%) Large number of events, small confidence intervals, appropriate statistics	High heterogeneity among studies, I^2 = 83%, 16 studies (6 retrospective) Different imaging methods (ultrasound, computed tomography, magnetic resonance imaging), lack of patient level data, not adjusted extensively for confounders. General limitation of epidemiological observational studies is cannot infer causality
NAFLD and modification in CVD risk assessment		
Framingham risk scores [57] (FRS) effectively predict CVD risk in 309 NAFLD patients followed for 11.5 years The MESA study [16] examined the impact of NAFLD diagnosed by computed tomography on non-fatal coronary heart disease and total mortality in 4119 individuals (17.6% with NAFLD) for 7.6 years. 209 individuals developed clinical events or died	FRS predicted CVD risk similarly in NAFLD and non-NAFLD patients suggesting that NAFLD does not modify risk prediction NAFLD resulted in an independent increased risk for clinical events hazard ratio: 1.42 (95% CI: 1.00 to 2.03) after adjustment for risk factors and coronary artery calcium	Small number of patients and lack of an independent replication cohort Risk was of borderline significance, and presence of NAFLD did not change risk discrimination (area under the ROC curves 0.7594 and 0.7593 respectively with and without NAFLD). The study did not test NAFLD potential to reclassify risk

CVD-cardiovascular disease; MESA multiethnic study on atherosclerosis; OR-odds ratio; ROC receiver operating characteristic curve.

benefit of a more intensive LDL-C lowering therapy with the simvastatin ezetimibe combo in comparison with simvastatin alone. However, the study was limited by its *post-hoc* design, and by the absence of imaging and histological data.

The Multi-Ethnic Study of Atherosclerosis (MESA) prospectively evaluated 4119 individuals without previous CVD, who had computed tomography liver scan to assess NAFLD, for a median of 7.6 years [16]. As expected, those with NAFLD (17.6%) had a greater prevalence of hypertension (53% *vs.* 46%) and T2D (22% *vs.* 11%). After adjustment for traditional risk factors and subclinical coronary atherosclerosis burden (i.e. coronary artery calcification, a robust marker of CVD risk [59]), those with NAFLD had a borderline higher risk of non-fatal coronary heart disease and total mortality (HR: 1.42, 95% CI: 1.00 to 2.03). However, the presence of NAFLD did not change risk discrimination (area under the ROC curves were similar 0.7594 and 0.7593 respectively with and without NAFLD). In this study, the potential of NAFLD to reclassify risk was not examined.

6.2. Does NAFLD presence indicate the need for subclinical vascular and cardiac disease evaluation?

Another important clinical question is if NAFLD presence indicates the need of a more extensive cardiovascular risk evaluation independently of the presence of classical risk factors. Indeed, many cross-sectional studies encountered independent associations between NAFLD and the presence of subclinical vascular disease like coronary artery calcification, elevated carotid intima media thickness and aortic stiffness or changes in heart morphology such as left ventricular hypertrophy or diastolic dysfunction, all being independent biomarkers of elevated CVD risk [2,60]. Indeed, an elevated coronary artery calcification burden helps identify higher risk for CVD individuals among T2D individuals [61], a group of patients where NAFLD is extremely prevalent [4]. However, there is no prospective evaluation showing an additional role of these imaging tests in CVD risk evaluation. Therefore, there is not enough evidence to routinely recommend imaging tests for subclinical vascular or heart disease based on the presence of NAFLD *per se*.

7. Treatment of NAFLD and cardiovascular prevention

7.1. Dietary changes, weight loss and physical activity

Correction of metabolic risk factors by lifestyle changes still represents the main treatment of NAFLD. Diets limited in calories, fructose and, alcohol restriction are recommended for individuals with NAFLD, especially NASH [5]. Furthermore, coffee consumption may reduce the risk of fibrosis progression, as it has been suggested by a meta-analysis of observational studies [62].

Robust evidence indicates that reduction of body weight (≥5%), independently of how it is achieved, improves liver damage in overweight patients with NAFLD. The impact is larger in those achieving ≥10% of weight loss [63,64]. Similarly, in severely obese individuals, bariatric surgery improves liver damage in most patients when significant weight loss is achieved [65]. On the other hand, sarcopenia and lack of physical activity are risk factors for NAFLD [66,67]. Physical exercise, acting by increasing energy expenditure, and leading to weight loss, can reduce intrahepatic triglyceride content [68]. In addition, gain in muscle mass, and the release of myokines and other mediators, confers protection from NAFLD independent of weight loss [69]. However, most NAFLD patients do not achieve weight loss, and even so this is not maintained in the long-term [70].

7.2. Pharmacological therapies to treat NAFLD and prevent cardiovascular disease

7.2.1. Acetylsalicylic acid (ASA)

ASA is a mandatory treatment for patients with symptomatic atherosclerotic cardiovascular disease (secondary prevention). However, there is controversy on the benefit of ASA use in primary prevention. A potential rational for the use of ASA in patients with NAFLD comes from a cross sectional study where individuals using this medication had lower levels of liver fibrosis indexes [71]. However, this study was likely biased as low platelets levels are the major indicator of advanced liver fibrosis and a contraindication for ASA administration. Therefore, use of ASA for CVD prevention in patients with NAFLD should follow recent recommendations based on CVD, colorectal cancer and bleeding risks [72].

7.2.2. Renin angiotensin inhibitors

The high prevalence of hypertension, T2D and kidney disease in individuals with NAFLD points to the activation of renin angiotensin system (RAS) as one of these mechanisms driving liver fibrosis. A recent study [73] observed an increased expression of components of the RAS and extra cellular matrix in animals developing liver steatosis. Experimental data and studies in humans suggest that the use of RAS antagonists reduces NAFLD progression in hypertensive patients [74]. In a recent observational study performed in 118 patients with NAFLD and followed by a median of 36 months, the use of RAS inhibitors (angiotensin converting enzyme inhibitors or angiotensin receptor blockers) was associated with lower histological fibrosis progression rates in diabetic patients [75]. However, randomized studies are necessary to prove the effects of RAS inhibition on NAFLD progression. Currently,

the prescription of RAS antagonists is the first line treatment for hypertension in diabetic patients, especially in those with early kidney disease, who are frequently affected by NAFLD.

7.2.3. Lipid lowering medications

NAFLD is also characterized by an increase in hepatic free cholesterol, which correlates with liver damage severity [76,77]. Remarkably, cross-sectional epidemiological studies have linked statin use with reduced risk of NAFLD [78], although evidence is still controversial [79]. Unfortunately, statins and ezetimibe have been tested as NASH treatment in grossly underpowered, or not controlled trials, or in studies lacking histological assessment of histological severity of liver damage [80]. In these studies, statins were well tolerated, reduced cardiovascular risk [81,82], and improved liver enzymes in at-risk patients [83], but results on liver-related outcomes were not conclusive [83–87]. Recent cross-sectional data in a large cohort of individuals at risk for NASH are also consistent with a possible protection of statin use on progressive liver disease, especially in patients negative for the PNPLA3 I148M mutation [88]. Indeed, statin therapy should not be precluded to individuals with a diagnosis of NAFLD, high cardiovascular risk, and elevated aminotransferases (usually > 3 times the upper limit of normal), since there is no evidence that statins will induce liver damage in this situation [89]. In these individuals, liver enzymes even might be reduced by statin treatment as it has been shown in *a post-hoc* analysis of the Greek Atorvastatin and Coronary Heart Disease Evaluation (GREACE) study [83].

Omega-3 fatty acids decrease lipogenesis and activate cellular receptors with anti-inflammatory activity [90]. Overall evidence suggests that they may improve dyslipidemia and decrease liver enzymes [91], with a larger effect in individuals negative for the *PNPLA3* mutation [92,93], but the impact of liver damage remains uncertain [94].

7.2.4. Insulin sensitizers

Since insulin resistance plays a major role in NAFLD pathogenesis, several drugs approved for T2D have been examined as potential treatments for liver damage in NAFLD patients. The effect on hepatic fat and fibrosis, the main determinants of NAFLD prognosis [19,20], is of utmost importance, since most NASH patients have T2D.

Metformin is still the cornerstone of T2D treatment. Despite initial encouraging results [95], metformin did not improve histological liver damage in patients without or with stable control of T2D [63]. However, in cross-sectional or retrospective studies, metformin has consistently been associated with protection from hepatocellular carcinoma development (while insulin with higher risk) [96], and with lower mortality in patients with cirrhosis [97]. Even if this evidence does not come from randomized controlled trials, it seems then advisable not to stop metformin in NAFLD patients with advanced liver disease, unless contraindicated by severe hepatic or kidney failure.

Glitazones, drugs acting on adipose tissue IR and adiponectin release [98], gave more promising results. However, despite these drugs induce amelioration of hepatic fat accumulation [99], the long-term impact on inflammation, especially fibrosis, is more doubtful, at the price of weight gain and other side effects. For example, in the PIVENS (Pioglitazone *versus* Vitamin E *versus* Placebo for the Treatment of Nondiabetic Patients with Nonalcoholic Steatohepatitis) trial, the lipophilic antioxidant vitamin E was more effective than pioglitazone in resolving inflammation [100]. Recent data however suggest that long-term treatment with Pioglitazone leads to improvement of liver damage, including fibrosis [101].

To conclude, there is consensus that patients with NAFLD must be encouraged to lifestyle changes, to reduce weight and increase physical activity. Metformin, lipid lowering medications (mainly statins) and RAS inhibitors are safe, well tolerated in individuals with NAFLD and the use of these drugs is highly desirable to reduce their cardiovascular risk when dyslipidemia, diabetes and hypertension are present. Moreover, the use of these drugs may lead to lower liver damage in individuals with IR and NAFLD. Inhibition of lipogenesis by omega-3 supplementation and statins may be particularly beneficial in those negative for the PNPLA3 mutation [88,92], but additional studies are required to confirm these findings that foresee the beginning of precision medicine.

8. Future therapies

The absence of a drug approved specifically for NAFLD treatment has led to a rapid evolution in the field of pharmacological therapy of this disease. The first phase III studies, evaluating the impact of two different nuclear receptor agonists, namely obeticholic acid and elafibranor, on histological damage, liver-related outcomes and survival in NASH patients, have just begun. Obeticholic acid is a potent agonist of Farnesoid X Receptor (FXR), with activity against hepatic fat accumulation, inflammation and fibrosis [102]. Obeticholic acid has been shown to reduce hepatic fat, histological activity and fibrosis in patients with NASH included in the phase IIB FLINT (Farnesoid X Receptor Ligand Obeticholic Acid in NASH Treatment) trial [103]. However, despite the concomitant use of statins, obeticholic acid use was associated with increased levels of LDL-cholesterol [103], and possibly insulin resistance worsening. Therefore, whether increased LDL-C represents a class effect of all FXR agonists, and if improving liver damage increases cardiovascular risk will be determined in the phase III REGENERATE trial (NCT02548351) and the upcoming studies with other new molecules belonging to this class.

Elafibranor is a peroxisome activated receptor (PPAR) alpha and delta agonist, which ameliorates liver damage in the most severe NASH patients included in the phase IIB GOLDEN-505 trial [104]. Despite the possibly less marked effect on reducing liver damage, elafibranor has shown beneficial effect on several metabolic syndrome traits (glucose, lipids and inflammation) [105]. The overall benefit of this drug is currently under investigation in the phase III RESOLVE-IT study (NCT02704403).

In the phase II LEAN study, the GLP-1 agonist liraglutide increased the rate of NASH resolution and reduced liver fibrosis progression, which was associated with weight loss in patients exposed to the drug [106]. On the same token in the LIRA-NAFLD study, liraglutide 1.2 mg/day induced an average 31% reduction in liver fat content after 6 months in uncontrolled T2D patients in comparison with insulin treatment [107]. Benefits were mostly dependent on weight loss. On the other hand, the DPP-4 inhibitor sitagliptin was not effective in reducing liver fat in diabetic patients [108].

More molecules are under evaluation in preclinical and phase I-II clinical studies [109]. These include several FXR and PPAR agonists, and molecules that inhibit inflammation, apoptosis, fibrosis deposition and stabilization. Their detailed description is outside the scopes of this review. However, it deserves to be mentioned that among these there are new classes of molecules such as fibroblast growth factor-21 analogues (FGF-21) or 19 (FGF-19) agonists, inhibitors of hepatic *de novo* lipogenesis, such as aramchol [110] or NGM282 [111], and SGLT2 inhibitors, with potential beneficial effects on both hepatic and metabolic correlates of NAFLD.

The future challenge is to understand the long-term impact of these new therapies on the risk of both hepatic and cardiometabolic hard endpoints. It is envisaged the possibility of associating several molecules with complementary actions. Ultimately, the goal is to identify subgroups of patients with high response to specific treatments.

9. Conclusions: gaps in current knowledge, call to action and future perspectives

Fig. 2 (graphical abstract) summarizes the controversies about the independent role of NAFLD as a cause of atherosclerotic cardiovascular disease. NAFLD is highly prevalent, especially in obese and diabetic individuals, and is associated with metabolic, inflammatory,

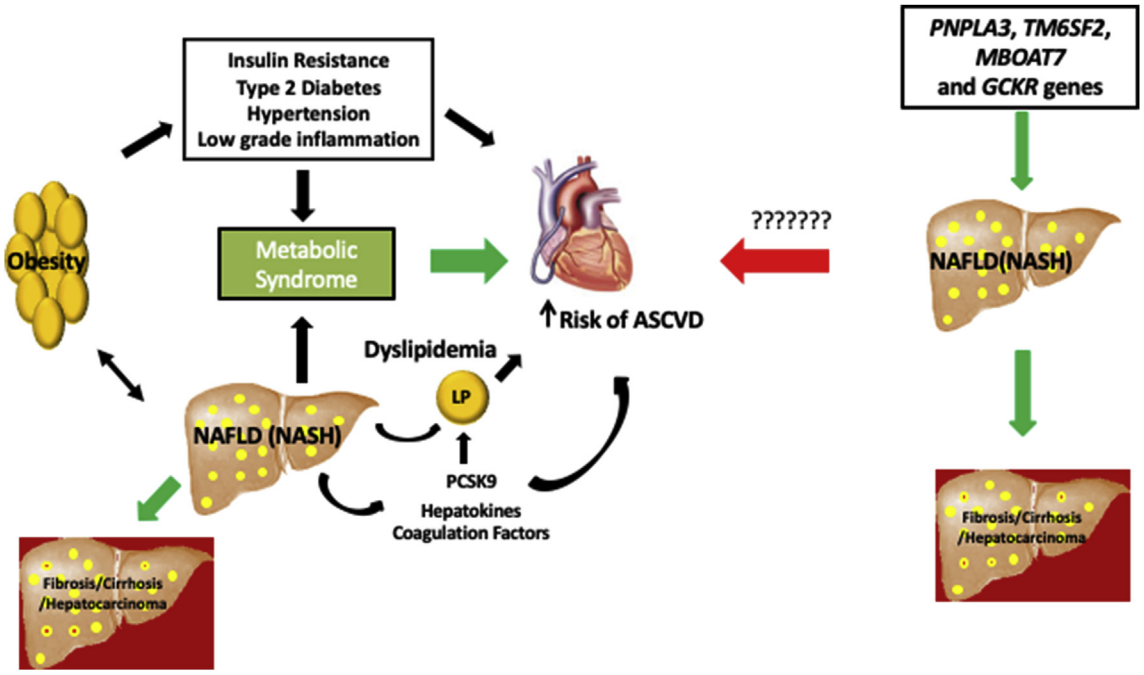

Fig. 2. Summary of controversies of NAFLD as an independent cause of atherosclerotic cardiovascular disease.
NAFLD is very frequent due to the obesity epidemics, and is closely associated with the metabolic syndrome. Both visceral and liver adipose tissues contribute to pro-atherogenic metabolic, inflammatory and coagulation abnormalities. These mechanisms play a causal role in atherosclerotic cardiovascular disease. NAFLD can evolve from simple steatosis to liver inflammation, fibrosis, cirrhosis and hepatocarcinoma. However, genetic variants that predispose to NAFLD development have not been associated with atherosclerotic CVD development in the absence of overall obesity and the metabolic syndrome. These variants are indeed associated with NAFLD progression and hepatic disease. LP- lipoproteins (VLDL); ASCVD: atherosclerotic cardiovascular disease; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis.

coagulation and blood pressure disturbances. Prospective studies have associated NAFLD presence and severity, particularly NASH and fibrosis, with an elevated risk of cardiovascular disease. However, these studies are limited by their heterogeneity and the presence of concomitant confounding factors. Therefore, there is still a need to prove NAFLD pathophysiologic role on atherosclerotic cardiovascular disease, independently of presence of the metabolic syndrome. In addition, it is important to better clarify how NAFLD progression from steatosis to more severe disease influences the metabolic and inflammatory components that may associate this disease with atherosclerosis. Genetic variants primary predisposing to NAFLD shed some light on the understanding of the relationship between NAFLD and atherosclerosis, suggesting this relationship being of epidemiological nature. Unfortunately, animal models based on these genetic variants were not able to explore the full spectrum of the disease and its association with atherosclerotic cardiovascular disease [112]. Furthermore, evidence has yet to be provided that NAFLD diagnosis can be used as a tool to better stratify cardiovascular risk and modify cardiovascular treatment.

Further studies are therefore necessary to demonstrate, for the vascular specialist, how NALFD diagnosis could influence clinical practice. Prospective studies aiming at demonstrating an association of NAFLD and its severity with the presence and evolution on markers of subclinical vascular disease like aortic stiffness or coronary plaques or calcification on CT angiography would help better understand if it has an independent role on vascular disease development.

Prospective long-term studies with homogeneous diagnostic criteria, considering not only the presence, but also the severity of NALFD are necessary to test if this diagnosis can improve cardiovascular disease risk stratification. The greatest challenge would be to separate it from its aggravating metabolic consequences that characterize the metabolic syndrome, like atherogenic dyslipidemia. Finally, specific drugs that are being developed for NAFLD must be tested prospectively in double blind studies in well-defined populations, possibly by liver imaging, to enroll adequate numbers of individuals with more severe forms of the disease e.g. NASH and fibrosis to determine the potential of reducing both hepatic and cardiovascular disease endpoints. With that, one may prove whether NAFLD is indeed a cause of atherosclerosis. In the meantime, considering the associated higher cardiovascular risk weight loss, exercise and control of concomitant established risk factors for atherosclerosis are mandatory in individuals with NAFLD.

Conflicts of interest

RDS has received honoraria for consulting, research and speaker activities from: Amgen, Astra Zeneca, Akcea, Biolab, Kowa, Esperion, Merck, Novo-Nordisk, Pfizer and Sanofi/Regeneron.

SR has consulted for Astra Zeneca, GSK, Pfizer, Amgen, Sanofi, Chiesi, Novo Nordisk and AKCEA therapeutics.

LV has nothing to declare.

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