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NAFLD OR MAFLD DIAGNOSES AND CARDIOVASCULAR DISEASES: FROM EPIDEMIOLOGY TO DRUG APPROACHES

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Background: A consensus of experts has proposed to replace the term nonalcoholic fatty liver disease (NAFLD), whose global prevalence is 25%, with metabolic dysfunction-associated fatty liver disease (MAFLD), to describe more appropriately the liver disease related to metabolic dysfunction. MAFLD is closely intertwined with type 2 diabetes, obesity, dyslipidemia, all linked to a rise in the risk of cardiovascular disease (CVDs). Since controversy still stands on whether or not NAFLD/MAFLD raises the odds of CVD, the present review aims to evaluate the impact of NAFLD/MAFLD etiologies on CV health and the potential correction by dietary and drug approaches.

Results: Epidemiological studies indicate that NAFLD raises risk of fatal or non-fatal CVD events. NAFLD patients have a higher prevalence of arterial plaques and stiffness, coronary calcification, and endothelial dysfunction. Although genetic and environmental factors strongly contribute to NAFLD pathogenesis, a Mendelian randomization analysis indicated that the *PNPLA3* genetic variant leading to NAFLD may not be causally associated with CVD risk. Among other genetic variants related to NAFLD, *TM6SF2* appears to be protective, whereas *MBOAT7* may favor venous thromboembolism.

Conclusions: NAFLD is correlated to a higher CVD risk which may be ameliorated by dietary interventions. This is not surprising, since new criteria defining MAFLD include other metabolic risk abnormalities fueling development of serious adverse extrahepatic outcomes, *e.g.*, CVD. The present lack of a targeted pharmacological approach makes the identification of patients with liver disease at higher CVD risk (*e.g.*, diabetes, hypertension, obesity or high levels of C-reactive protein) of major clinical interest.

Keywords: cardiovascular risk, MAFLD, NAFLD, PNPLA3, PCSK7, PCSK9, TM6F2, MBOAT7

1. Introduction

Over the past two decades, nonalcoholic fatty liver disease (NAFLD) has raised the burden of death and disability caused by cardiovascular diseases (CVDs) [1]. The prevalence of NAFLD in adults is 25% (95% CI: 22.10-28.65) worldwide, the highest being reported in the Middle East (32%) and South America (31%), followed by Asia (27%), USA (24%) and Europe (23%), with the lowest in Africa (14%) [2,3]. Considering that NAFLD and CVD share common risk factors such as atherogenic dyslipidemia, insulin resistance (IR), and hypertension, the overall aim of the present review is to cover aspects common to the two conditions, along with the description of new pharmacological approaches to NAFLD that could ameliorate CVD risk. However, due to the very recent recommendation to switch the terminology NAFLD into metabolic dysfunction-associated fatty liver disease (MAFLD), this aspect has been taken into consideration in the research strategy and throughout the manuscript. By using the Pubmed database, the following algorithm has been used: *NAFLD* OR *MAFLD* AND *adipose tissue* AND *alcohol consumption* AND *cardiovascular disease* AND *dietary approach* AND *ectopic fat* AND *epigenetic* AND *genetic determinants* AND *inflammation* AND *insulin resistance* AND *pharmacological treatment* AND *subclinical atherosclerosis AND type 2 diabetes mellitus.* Relative to clinical studies the search for literature comprised observational, retrospective, interventional and prospective studies. PD and MR screened titles and full text of papers identified in our search.

Although not in the remit of the present review article it is worth mentioning that NAFLD is also associated with an increased risk of other cardiac complications (valvular calcification, left ventricular hypertrophy and certain arrhythmias) independent of common CVD risk factors [4].

NAFLD associates also with a raised risk of liver-related morbidity or mortality, and is considered a multisystem disorder, affecting a variety of extra-hepatic organs, including the CV system [5]. The largest meta-analysis (n= 34,043 adult individuals) of observational, prospective and retrospective studies indicated that patients with NAFLD have an odds ratio (OR) of 1.64 (95%CI 1.26-2.13) for fatal and/or non-fatal CVD events. This risk increases stepwise with the progression of the disease, leading to an OR of 2.58 (95%CI 1.78-3.7%) in patients with the most severe NAFLD phenotype [6]. In a similar way, a closer link between NAFLD and subclinical atherosclerosis has been reported: the presence of NAFLD raises the odds of carotid-intima

media thickness (c-IMT), arterial stiffness and coronary artery calcification by 1.74 (95%Cl 1.47-2.06), 1.56 (95%Cl1.24-1.96), and 1.40 (95%CI 1.22-1.60), respectively [7].

2. Metabolic fatty liver disease (MAFLD) – a novel acronym

Several years ago, concerns over the accuracy of the fatty liver disease nomenclature were raised [8], encouraging renaming this definition. Indeed, a consequence of using inappropriate terminology may generate mistrust between patients and clinicians and most importantly terminology that contains the term "non" may diminish the importance of a disease leading to a false perception[9]. NAFLD terminology can be considered as just an umbrella term, essentially targeting a single feature, *i.e.*, hepatic steatosis. It is a condition of "exclusion" since it exists only in the absence of conditions such as viral hepatitis, autoimmune disease or alcohol intake. Conversely, manifestation of fatty liver should be the final sum of different interactions, from circulating lipids, to IR to genetic background [10]. The term NAFLD could be an element of confusion since the use of "non" and of words such as "alcoholic" is disliked by patients leading to a fear of stigma, especially in pediatric conditions [11]. All these criticisms have been the cue to find a terminology addressing the heterogenous clinical presentation of the disease. In 2020, a consensus of experts has proposed to replace the term NAFLD with a more appropriate one, *i.e.*, MAFLD [12]. This new nomenclature should allow to properly stratify patients via the application of more precise genetic, anthropometric, and metabolic approaches. This new definition clearly establishes this disease as a metabolic disorder. As depicted in Figure 1, to meet the diagnosis of MAFLD, patients require the presence of hepatic steatosis (histology, imaging, blood markers or scores of evidence of fat accumulation) accompanied by one of the following three features: overweight or obesity (cut-offs according to the ethnicity), type 2 diabetes mellitus (T2D) or signs of metabolic dysregulation. This last is defined as the presence of two or more conditions: (1) enlarged waist circumference; (2) raised blood pressure or specific drug treatment; (3) raised triglycerides (TG) or specific drug treatment; (4) low high-density lipoprotein cholesterol (HDL-C); (5) prediabetes; (6) high Homeostatic Model Assessment of IR (HOMA-IR) score; (7) inflammation with raised levels of high-sensitivity C-reactive protein (hsCRP) [12]. Overall, the ability of these non-invasive scoring models to discriminate between NAFLD and MAFLD has to be validated in future studies [13], as in the case of serum biomarkers of fatty liver that could replace imaging

methods. Currently, should the epidemiological studies be conducted or analyzed again, the former assumption appears to be valid for markers with a high prognostic score, *e.g.*, fatty liver index [14].

This new definition is challenging and probably could lead to different conclusions when applied to previous studies. On this matter, a recent study on 756 Japanese patients with fatty liver demonstrated that the application of the MAFLD criteria identifies individuals with fatty liver and significant fibrosis, with a higher sensitivity for detecting significant fibrosis in MAFLD than NAFLD, *i.e.*, 93.9% and 73.0%, respectively [15]. Similar conclusions have been reached when the two definitions have been used to characterize 13,083 cases with complete ultrasonographic and laboratory data. Compared with those with NAFLD, patients diagnosed as MAFLD were more likely to have multiple metabolic comorbidities with more frequent advanced fibrosis [16]. Moreover, as highlighted by Targher, while these real-world data show an excellent concordance between MAFLD and NAFLD (Cohen's kappa coefficient of 0.92), the two terms are not mutually interchangeable and do not identify exactly the same individuals [17].

3. Risk factors contributing to NAFLD/MAFLD

Considering the multifactorial etiology of fatty liver, the next sections will address the contribution of genetic and epigenetic factors, ethnicity, age, sex, body composition, dietary habits, as well as changes in microbiota composition on the development of NAFLD/MAFLD. As reported by the International expert consensus statement, MAFLD could take into consideration, in the future, also pathophysiological modifiers leading to the development of a morphologically limited set of histological features. This could be the presence of the genetic variants for Patatin-like Phospholipase Domain–Containing 3 (*PNPLA3*), Transmembrane 6 Superfamily Member 2 gene *(TM6SF2)*, Membrane Bound O-acyltransferase Domain-containing 7 (*MBOAT7*) and Hydroxysteroid 17-Beta Dehydrogenase 13 (*HSD17B13*), as well as epigenetic and or other disease modifiers[14].

3.1 Genetic contribution. NAFLD is a complex disease whose pathogenesis and progression result from gene-environment interactions [18]. Familial, twin and epidemiological studies indicate that NAFLD has a strong inherited component, ranging from 20 to 75% depending on ethnicity,

environmental factors and methodology. Family members of overweight children with NAFLD have a higher susceptibility to develop fatty liver compared to family members of obese children without NAFLD [19]. In 313 Finnish twins, ~60% of the variation in serum alanine aminotransferase (ALT), a marker of liver fat content, was genetically determined [20]. The concept of inheritance was further confirmed by Loomba et al who reported that the presence of hepatic steatosis and fibrosis was correlated between monozygotic but not between dizygotic twins [21]. Conversely, a negligible role for the heritability of NAFLD was found in a cohort of 208 adult Hungarian twins (63 monozygotic and 41 dizygotic pairs); in this series NAFLD was associated with carotid plaque formation and raised c-IMT [22].

Single nucleotide polymorphisms (SNPs) in genes regulating the hepatic lipid handling have been broadly associated with increased susceptibility to develop the entire spectrum of NAFLD, from steatosis, to nonalcoholic steatohepatitis (NASH) and fibrosis (Figure 2). Hepatic fat accumulation represents the main driver of the progression to end-stage liver damages in genetically predisposed individuals and the effect of each genetic variation on the spectrum of NAFLD is closely intertwined with the ability to induce fat storage [23]. The dominant genetic modifiers known to shape both NAFLD susceptibility and progression are the variants in *PNPLA3*, *TM6SF2*, *MBOAT7*, Glucokinase regulator (*GCKR)* and *17β-HSD13* genes (Figure 3).

3.1.1 PNPLA3. The rs738409 C>G polymorphism in the *PNPLA3* gene, encoding the aminoacid substitution of isoleucine to methionine at position 148 (p.I148M), accounts for the largest fraction of genetic predisposition to NAFLD and for the entire spectrum of related progressive liver damage [24,25]. It was identified in 2008 by a genome-wide association study (GWAS) evaluating a North American population of different ethnicities (Dallas Heart Study). The prevalence of the G at-risk allele was higher in Hispanics (49%) than in Europeans (23%) and less frequent in African Americans (17%), thereby explaining the inter-ethnic susceptibility to NAFLD [26]. PNPLA3 is mainly localized in the endothelial reticulum (ER) and at the lipid droplet surface in hepatocytes, adipocytes and hepatic stellate cells (HSCs) where it exerts a hydrolyzing activity towards TG and retinyl esters [27]. While the wild-type protein is rapidly degraded, the mutated form escapes ubiquitination and accumulates on lipid droplets, thus impairing TG mobilization by other lipases (ATGL/PNPLA2), as well as turnover and catabolism [28]. Another potential

mechanism explaining TG accumulation in the presence of the p.I148M variant is impaired lipophagy in hepatocytes. This mechanism may hamper autophagic fluxes and lipid droplet degradation [29].

A lipidomic analysis demonstrated that TG in very-low density lipoproteins (VLDL) were depleted of polyunsaturated fatty acids (PUFAs) in I148M homozygotes both under fasting and postprandial conditions [30]. By using *in vitro* models of I148M hepatic cells, the same authors demonstrated a raised PUFA incorporation into TG, whilst PUFA-containing diacylglycerols (DAGs) accumulated at the expense of phosphatidylcholines [30]. The hepatic lipid composition of DAG species may affect insulin sensitivity, although the alteration in liver DAG levels was not confirmed by Franko et al. who concluded that the *PNPLA3* variant is tightly associated with fatty liver, but not with IR, thereby dissociating these two features [31].

Another intriguing aspect regards ceramide synthesis, a process identified as the key mediator of hepatic IR. Since ceramide enriched liver lipidome was observed in IR-related NAFLD but not in *PNPLA3* NAFLD, this may explain why metabolic NAFLD and not PNPLA3-related NAFLD is tightly correlated with an increased risk of T2D and CV events [32].

Furthermore, the I148M variant impairs retinol release from HSCs, directly participating in fibrogenesis and carcinogenesis, independent of predisposition to fatty liver [24]. NAFLD patients who carry the G allele are characterized by the activation of hepatic stem/progenitor cell (HpSC), associated with a more aggressive histological pattern (portal fibrogenesis) and oxidative stress [33].

3.1.2 TM6SF2. In 2014, an exome wide association study identified the rs58542926 C>T genetic variant in the transmembrane 6 superfamily member 2 gene (*TM6SF2*) as a determinant of hepatic TG content, serum aminotransferases, low-density lipoprotein cholesterol (LDL-C) and TG. It encodes the substitution of the loss-of-function lysine (E) with glutamic acid (K) at position 167 (E167K) [34]. TM6SF2 localizes in the ER and ER-Golgi compartments and participates in hepatic VLDL lipidation and assembly in the ER cisternae. The E167K variant causes a TM6SF2 misfolded protein undergoing rapid intracellular turnover and degradation thus leading to hepatic down-regulation [34].

The E167K genetic variant is independently associated with higher circulating levels of ALT, hepatic TG content, and NAFLD stages in both children and adults [35,36]. Another exome-wide association study showed that, among 444 coding and noncoding genetic variants associated with plasma lipids, the E167K *TM6SF2* variant was related to an increased risk of fatty liver and T2D [37]. In line with these findings, in a large cross-sectional cohort of 1,201 individuals with biopsy-proven NAFLD, the E167K variant was associated with a higher degree of steatosis, necroinflammation, ballooning and fibrosis, but was protective against CV events [36]. Therefore, it has been suggested that the T risk allele may disentangle NAFLD from CV disorders, while increasing liver disease severity [36,38].

Liver lipidome analyses in NAFLD carriers of the E167K variant showed that hepatic TG and cholesteryl ester (CE) were higher, whereas phosphatidylcholines (PCs) were lower. In addition, incorporation of PUFA into TG and PC in *TM6SF2* knockdown hepatocytes was reduced, suggesting that impairment in liver lipid synthesis from PUFAs could contribute to a deficiency in PCs and increased intrahepatic TG in E167K *TM6SF2* carriers [39].

3.1.3 MBOAT7. In 2015, a GWAS found that the common rs641738 C>T variant in the *MBOAT7-TMC4* locus on chromosome 19 increased susceptibility to cirrhosis in alcoholics [40]. Mancina and Dongiovanni further demonstrated that the rs641738 variant associates with liver fat accumulation and to the whole phenotypic spectrum of liver injuries related to NAFLD [41], as also confirmed in pediatric NAFLD [42,43].

MBOAT7, known as lyso-phosphatidylinositol (lyso-PI) acyl-transferase1 (LPIAT1), encodes an enzyme member of the Lands' cycle of phospholipid acyl-chain remodeling in membranes. It is mainly localized in the membrane bridging ER and mitochondria where fat biosynthesis and lipid droplet formation occur. MBOAT7 conjugates an acyl-CoA to the second acyl-chain of lyso-phospholipids, using arachidonoyl-CoA as the substrate. Thus, it modulates desaturation of phospholipids and availability of free arachidonic acid, the precursor of proinflammatory eicosanoids. These mechanisms underlying the association between the rs641738 variant and liver damage are related to the hampered hepatic gene and protein expression of MBOAT7, thus altering the phosphatidylinositol species [41,44 45]. An impairment in MBOAT7 function contributes to the accumulation of saturated phospholipids, mainly phosphatidylinositol species that may be

shunted to the synthesis of saturated and mono-unsaturated TG, further contributing to fatty-laden hepatocyte formation [44,46].

***3.1.4 GCKR*.** Alongside *PNPLA3*, *TM6SF2* and *MBOAT7* variants, even the common loss-of-function rs1260326 C>T variant in the *GCKR* gene (c.1403C>T, p.P446L) has been associated with increased fasting TG concentrations, large VLDL, steatosis and liver damage [47]. The *GCKR* gene codifies for the GCKR protein, that exerts a crucial role in glucose homeostasis regulating glucose influx into hepatocytes and activating *de novo* lipogenesis (DNL).

Santoro et al. reported for the first time the rate of DNL by determining incorporation of deuterium into palmitate in VLDL after administration of a carbohydrate drink (75 g glucose and 25 g fructose) in obese adolescents. In these individuals, the *GCKR* rs1260326 variant in homozygosity increased liver lipid synthesis as the result of an enhanced glycolytic carbon flux to TG formation. Moreover, the combination of *PNPLA3* and *GCKR* minor alleles may explain up to 32% of the liver fat content in Caucasian obese children, 39% in African-Americans and 15% in Hispanics [35]. Furthermore, this variant has been also associated with increased susceptibility to NASH, fibrosis and hepatocellular carcinoma (HCC) [48] in adult NAFLD patients, without altering the atherogenic lipid profile and the CV risk [49].

3.1.5 HSD17B13. In 2018, an exome-wide sequencing identified the rs72613567 variant in the hydroxysteroid 17-beta dehydrogenase 13 (*HSD17B13*) gene, associated with protection against histological steatohepatitis, clinically significant fibrosis and cirrhosis, in both NAFLD and alcoholic fatty liver disease (ALD) [50]. The rs72613567 variation corresponds to an insertion of an adenine adjacent to the donor splice site of the last exon (TA allele), resulting in a truncated transcript, reduced expression and impaired enzyme activity of the HSD17B13 protein [51]. Hsd17b13 KO mice have an impaired hepatic-lipid metabolism, resulting in raised hepatic TG content, directly inducing DNL through sterol regulatory element-binding protein (SREBP)1 and fatty acid synthase [51,52].

The HSD17B13 protective effect is more relevant in the development of steatohepatitis than in progression to fibrosis [47]. The likely protective mechanism of the *HSD17B13* rs143404524 polymorphism appears to be an increased concentration of hepatic phospholipids that couples

with a down-regulation of pro-inflammatory genes [53]. Furthermore, the rs72613567 variant has been related to a reduced risk of elevated transaminases and HCC [54,55].

3.2 Allelic pleiotropy: how genetic variants related to NAFLD affect CV risk. A genetic variant may influence multiple traits through a mechanism defined as "allelic pleiotropy" [56]. A meta-analysis of 7,176 NAFLD individuals of European descent showed that the rs780094 T allele at GCKR was associated with higher levels of LDL-C, TG and increased 2 hours glycemia. The E167K *TM6SF2* variation was instead related to lower LDL-C and TG levels, whereas the I148M *PNPLA3* variant was not associated with any of these traits [57]. The conclusion that carriers of the rs738409 *PNPLA3* variant have a raised risk of NALFD progression but not of IR or metabolic syndrome highlights the conclusion that not "all forms of NAFLD were created equal" [58]. Conversely, an association between I148M *PNPLA3* (95%CI 1.03-1.07) and E167K *TM6SF2* (95%CI 1.05-1-12) polymorphisms and diabetes was found in a fine mapping study, which evaluated 81,412 T2D cases and 370,832 controls of diverse ancestry, further confirming the contribution of the *GCKR* P446L variant (OR 1.05; 95%CI 1.04-1.07) on IR [59].

Relative to coronary artery disease (CAD), a GWAS meta-analysis of ~185,000 cases and controls, showed an association of the *PNPLA3 G-allele* rs738409 with a modest protection from CAD, albeit significant only by running a recessive model (the odd was 0.92 (95%CI 0.87-0.97)) [60] (Table 1). Thus, it is tempting to speculate that a possible cardioprotective effect may be related to the reduced VLDL secretion, a consequence of the hampered breakdown of intrahepatic TG associated to the PNPLA3 I148M protein. Interestingly, in a cohort study in the Danish general population (n=94,708), the I148M (rs738409) was not causally associated with protection against ischemic heart disease (OR: 0.95; 95%CI 0.86–1.04), although ischemic heart disease increased stepwise with increasing liver fat content (OR: 2.41; 95%CI 1.28–4.51) [61]. However, it is worth mentioning that conflicting results have been also reported in the few observational studies that assessed the association between *PNPLA3* rs738409 SNP and evaluation of c-IMT [58].

The rs58542926 T-allele variant in the *TM6SF2* gene was also found to be protective against CAD [60] and a systematic evaluation of coding variants in >10,000 Norwegians revealed that the *TM6SF2* rs58542926 variation reduced total cholesterol and the incidence of myocardial infarction (OR= 0.87; 95%CI 0.79-0.95) [62]. Contrasting data have been provided in the case of

rs641738 T-allele (*MBOAT7*) and risk of CAD, *i.e.*, a neutral effect [60] or an increased risk of venous thromboembolism [63]. Finally, in 2017, Liu et al. tested the association of genotypes from the Human Exome Bead Chip with lipid levels and found that both *TM6SF2* rs58542926 and *PNPLA3* rs738409 variants were associated with lower lipid levels and a lower risk of CAD, but with an increased risk of steatosis and T2D [37]. Finally, the genetic variant rs641738C > T near *MBOAT7*, predisposing to raised hepatic fat, MAFLD and susceptibility to develop NASH, did not associate with CAD risk [64].

To sum-up, variants which represent the best genetic predictors of NAFLD are associated with reduced CAD risk or at least do not predispose to CAD. The epidemiological association between fatty liver and cardiovascular damage may be mostly mediated by dyslipidemia and IR, both classical risk factors for atherosclerosis. To definitively state whether the genetics of NAFLD can affect CVD, other variations predisposing to NAFLD without affecting lipid secretion and plasma cholesterol and TG should be considered.

3.3 Epigenetic programmes in NAFLD. Nowadays, genetic variants associated with NAFLD account for only a minor fraction of the overall heritability. Missing information may be attributed to rare variants, to common variants that have not reached genome-wide significance, or to epigenetic modifications (in particular miRNAs) [65,66].

An altered miRNA profile has been described in NAFLD and NASH, both in humans and experimental models [67]. Pirola et al explored the circulating miRNA signature associated with NAFLD revealing that, among 84 miRNAs analyzed, miR-122, miR-192, miR-19a/b, miR-125b and miR-375 were up-regulated in simple steatosis and, more so, the expression of miR-122, miR-192 and miR-375 in NASH, potentially distinguishing this condition from simple steatosis [68]. Differentially expressed miRNAs have been associated with hepatic fibrosis and progression to HCC. Cermelli et al. observed that circulating levels of miR-122, miR-34a and miR-16 were strongly correlated with liver enzymes, inflammatory activity and fibrosis score [69]. Expression of miR15 and miR16 is reduced in activated HSCs and, together with miR-34, they are also involved in the regulation of cell cycle progression, proliferation and hepatocarcinogenesis [70].

Epigenetic changes may play a role in the fetal programming of liver fat [71]. Elevation of ALT was associated with HIF3A methylation in children with NAFLD, thus suggesting that

epigenetic changes in the oxidative stress response predispose to fatty liver [72]. Maternal obesity and infant nutritional habits may be associated with methylation of the peroxisome proliferator-activator receptor γ coactivator-1 alpha (PGC1α) gene [73], a key regulator of mitochondrial biogenesis and fatty acid oxidation in NAFLD [74]. Acetylation patterns also play a role. *In utero* exposure to maternal high-fat diet increased fetal H3K14 histone acetylation with a parallel decrease in SIRT1 expression and histone deacetylase activity, linked to an altered expression of genes mediated by SIRT1 and involved in fatty acid oxidation and lipogenesis [75].

In patients with biopsy-proven NAFLD, the decreased expression of PGC1α was inversely related with its promoter methylation. The latter was also positively related with plasma fasting insulin and HOMA-IR, thus suggesting that the IR phenotype and epigenetic changes are tightly intertwined with fatty liver development [76]. The protective mechanism driven by PGC1α against hepatic steatosis and IR occurs by enhancing the IL-10 mediated anti-inflammatory response [77]. A novel epigenetic regulator of lipogenesis is hepatic Slug, a transcriptional factor which binds to the promoter of lipogenic genes fatty acid synthase (*FASN*), acetyl CoA Carboxylase 1 (*ACC1*), and Sterol regulatory element-binding transcription factor 1 (*SREBP1C*) **[77]**. Insulin raises the expression of Slug in primary hepatocytes, followed by binding to fatty acid synthase, thus stimulating the lipogenic program. Deletion of Slug inhibits lipogenesis and protects against NAFLD and obesity [78]. Alterations in the methylation signature of hepatic and peripheral blood-derived DNA, including major regulatory loci of metabolic inflammatory and fibrotic pathways, have been evaluated in NAFLD patients. Epigenome wide-association studies (EWAS) identified methylation changes in genes involved in liver function, cholesterol synthesis and steatosis development approximately explaining 10% of the interindividual variation [79]. The CpG island (CpG99) in the genomic region of *PNPLA3* was hypermethylated in NAFLD patients and CpG26 was hypomethylated, both possibly contributing to fibrosis severity [80].

Data on the role of long non-coding RNAs (lnc-RNAs) and other non-coding RNAs in NAFLD are still preliminary. The hepatic expression of Lnc1-8q22.2 [81] was significantly raised in patients with NASH, whereas that of lnc-RNA1 (*BLNC1*) was elevated in the liver of obese mice with NAFLD [82]. Liver specific inactivation of *Blnc1*, required for the induction of lipogenic genes, abrogated high-fat-diet-induced steatosis, IR and protected mice against NASH.

3.4 Ethnic differences. Reasons for racial disparities in the prevalence of NAFLD are not fully understood. Genetic heritage across ethnic groups may be associated with the prevalence of the *PNPLA3* at risk allele, occurring more frequently in Hispanic (49%) followed by non Hispanic whites (23%) and African American individuals (17%) [26]. Moreover, population-based data have shown a clear ethnic footprint in fatty liver prevalence. This appears to be highest, both for NAFLD and NASH, in Hispanic individuals, intermediate in Whites and lowest in Blacks, whereas fibrosis does not appear to differ according to ethnicity [83]. Fatty liver appears to be increasing in Asian populations with an apparent more severe course [84]. Asian individuals seem to have a higher risk of fibrosis, whereas this risk is lower in African individuals compared to whites [85].

3.5 Age and sex differences. These features have a definite influence on the likelihood of overall and disease specific mortality. Advancing age [86] leads to substantial changes in liver structure with reduced blood flow and volume, together with reduced bile acid synthesis and alterations in cholesterol metabolism. Aging also leads to changes in body composition, including decreased muscle mass, raised abdominal adiposity and development of IR [87]. Concerning sex-differences, as elsewhere extensively reviewed [88], the overall prevalence of NAFLD is higher in men than in women, and becomes similar after the age of 50-60 years [89]. On this matter, although no specific studies have been designed to assess sex differences in NAFLD, a large meta-analysis comprising 62,239 individuals with NAFDL showed that, compared to men, women had a 19% lower risk of NAFLD with a relative risk of 0.81 (95%CI 0.68-0.97), but once established, the risk of advanced fibrosis is higher in women especially after the age of 50 [90]. Interestingly, application of the MAFLD definition to a cohort of 756 Japanese with a previous diagnosis of NAFLD showed that MAFLD patients were more likely to be men [15]. In search of possible pathophysiological mechanisms explaining sex differences in NAFLD, it can be acknowledged the X chromosome dosage, significance of sex hormones, sex/gender-associated IR, as well as the distinct regional fat distribution and adipocyte biology [89]. Finally, considering that over 1,000 hepatic genes are differentially expressed between female and male livers, roles of peroxisome proliferator-α activated receptor (PPARα), farnesoid X receptor (FXR) and liver X receptor (LXR) regulatory factors have to be acknowledged [91].

3.6 Obesity. It can be classified as metabolically healthy and unhealthy, although individuals who are reported as having metabolically healthy obesity are often not truly healthy, but have fewer cardiometabolic abnormalities *vs* metabolically unhealthy individuals [92]. Overall, the metabolically unhealthy obese are at greater risk of T2D, CVD and all-cause mortality than those with metabolically healthy obesity [93]. Factors involved in the transition from metabolically healthy to metabolically unhealthy obesity are a prolonged excess adiposity, a decline in IR, a rise in fasting plasma glucose as well as the presence of NAFLD. This last condition worsens hepatic and systemic IR, favors atherogenic dyslipidemia and leads to the release of proinflammatory mediators [94]. A significant proportion of metabolically healthy obese display an altered fat distribution, confirming that a higher amount of visceral fat leads to a raised CV metabolic risk [95]. Visceral obesity is linked to enhanced inflammation and fibrosis, independent of IR and steatosis [96].

A number of favorable adiposity genes have been recently identified and may be crucial in predicting fibrosis and cirrhosis risk in overweight/obese individuals [97]. In contrast to obesity associated NAFLD, in lean individuals (BMI < 25 kg/m^2) without "significant" alcohol intake, NAFLD occurs more frequently in Asian populations. In this ethnicity between 5 and 45% of patients with NAFLD are lean [98], apparently with an accelerated disease progression.

***3.7 Alcohol consumption*.** The threshold of alcohol intake in NAFLD patients is 30 g/day for men and 20 g for women, below which it does not induce steatosis or exert damaging effects on liver disease progression [99]. This general consensus has led to the recent debate on the safe limits on alcohol consumption in the setting of NAFLD [100]. This debate has led to the conclusion that alcohol intake has likely a straight dose-response toxicity, rather than a J-shaped association with liver disease and with synergistic damaging effects in the presence of the metabolic syndrome. This supports the ongoing change in nomenclature [101,102], also improving the definition of the relationship with CHD [5].

4. NAFLD and risk of cardiovascular diseases

A number of reports have conclusively established that NAFLD is an independent CV risk factor, considering in particular the similarity in risk profile between NAFLD and the metabolic syndrome

[6,103,104]. As elsewhere reviewed [105], NAFLD associates with increased risks of myocardial infarction, subclinical coronary or carotid atherosclerosis, as well as of valvular heart disease and the magnitude of risk correlates to the severity of NAFLD [106]. Although no definite pathophysiological mechanisms explain this liaison, some hypotheses rely on hypertension, increased arterial stiffness [107], hyperuricemia, a rise in the hepatic production of multiple prothrombogenic factors, increased oxidative stress and adipose tissue inflammation, as well as an altered adipokine profile [108].

4.1 Lipid profile. The liver plays a central role in lipoprotein metabolism as it participates in the production and/or clearance of all classes of lipoprotein particles. As elsewhere reviewed [109], alterations in hepatic lipid metabolism that lead to NAFLD also drive the development of atherogenic dyslipidemia.

Elevated triglyceride is an almost constant finding in patients with NAFLD. In the presence of steatosis, TG elevations are in particular associated with coronary plaques, both calcified and non-calcified, as well as with non-obstructive coronary stenoses [110]. Besides being correlated *per se* with calcified plaques independent of the presence of the metabolic syndrome in NAFLD patients [111], higher TG and remnant cholesterol have been observationally and genetically associated with an increased risk of aortic valve stenosis [112]. Overall, the association between serum TG and hepatic steatosis is largely accounted for by a greater TG enrichment in VLDL particles [113]. On this matter, Boren et al have demonstrated by kinetic studies how $VLDL_1$-TG production is 35% lower in homozygous *TM6SF2 E167K* carriers [114]. This finding provides a basis for understanding the lower CV risk associated with this mutation [36] , associated with a rise in hepatic lipid droplet TG content [115] and predisposing to NAFLD [116].

TG can be a crucial factor in the progression from NAFLD to NASH. VLDL synthesis is impaired in NASH, possibly as the result of lipid oxidative DNA damage, leading to dysfunctional VLDL synthesis and lipid outflow as key factors in the progression to NASH [117]. In the context of TG-rich lipoproteins, fasting remnant-cholesterol, another CV risk factor, is associated with the odds of NAFLD beyond traditional risk factors, such as adiposity and IR [118]. Fatty liver and elevated TG can be finally linked to apoprotein C3 (*APOC3*) variant alleles (C482T, T455C or both). Among healthy Asian Indian men, carriers of these *APOC3* variants had a 30% increase in apoC3

levels [119], a 60% increase in fasting TGs and a 38% prevalence of fatty liver disease compared to wild type homozygotes. Similar conclusions were reached in a meta-analysis reporting that carriers of the genotype *APOC3* rs2854116 had a 45% higher risk to develop NAFLD [116]. ApoC3 is also involved in glucose homeostasis, monocyte adhesion, activation of inflammatory pathways and modulation of the coagulation cascade [120].

Besides the role played by TG-rich lipoproteins, altered levels of proatherogenic lipoprotein subclasses have been described, namely an increased percentage of small-dense (sd) LDL [121]. 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.

The significant reduction of HDL-C in NAFLD appears to be associated with an impaired HDL cholesterol efflux capacity [122]. This last appears to have an independent negative correlation with c-IMT as well as with the presence of atherosclerotic plaques [123], thus confirming the widely held view of a significant correlation between liver steatosis and CV risk. Significant apoAI reductions have been frequently reported in liver disease patients [124] with a significant rise after resolution of fatty liver in patients after weight reduction [125].

In the context of non-classical lipid biomarkers, the possible role played by both proprotein convertase subtilisin/kexin type 9 (PCSK9) (one of the key regulators of LDL-C) [126] and PCSK7 is worth mentioning. We previously demonstrated that the *PCSK7* rs236918 variant was associated with the severity of liver disease in biopsy-proven NAFLD patients, thus correlating with dyslipidemia and hepatic inflammation [127] . In the case of PCSK9, no definite conclusions have been reached. Although preclinical [128] and retrospective studies found a positive association with the severity of fat accumulation [129], genetic studies did not confirm this evidence [130,131], leading to the conclusion that PCSK9 inhibition may not be linked to an increased risk of NAFLD.

4.2 Insulin resistance. Several data demonstrated that IR is a key player in the development of NAFLD and its progressive forms [132]. Indeed, IR correlates with the severity of liver fibrosis, the main determinant of NAFLD prognosis, and advanced fibrosis is often observed in T2D patients with NAFLD [133]. Moreover, genetic variants that dampen insulin receptor (InsR) signaling may favor fibrosis development in NAFLD [134]. IR also creates a pro-atherogenic environment for CVD development, *i.e.*, by favoring dyslipidemia, hyperglycemia, activation of oxidative stress,

endothelial dysfunction, and ectopic lipid accumulation [135]. What is clear today is that human genetic variations primarily increasing liver fat content do not have a direct effect on IR. It is the quality of fat rather than the quantity that causes IR [136].

The molecular activation of lipogenesis in liver steatosis may be consequent to a raised glucose production, followed by conversion to fatty acids via pyruvate, entering the Krebs cycle [137]. This synthesis, regulated by insulin, is mediated by the membrane bound transcription factor *SREBP1c*, not appropriately activated in the presence of IR. *SREBP1c* can activate the transcription of PPARγ, a nuclear receptor required for normal adipocytes to achieve differentiation and involved in hepatic steatosis development. Deletion of PPARγ in different models leads to reduced steatosis independent of hyperinsulinemia and hyperglycemia [138]. Activation of PPARγ, in contrast, leads to raised insulin secretion [139] and may worsen steatosis, although clinical trials with the PPARγ agonist rosiglitazone have shown improved liver steatosis [140]. Finally, AMP activated protein kinase (AMPK) stimulates fatty acid β activation and lipogenesis [141]. Activation of AMPK occurs after the antidiabetic metformin, markedly reducing steatosis [142] and liver size in man [143].

4.3 Adipose tissue, ectopic fat accumulation and inflammation. Adipose tissue, beyond its role as a fat storage depot, is an endocrine organ, capable of producing and releasing biologically active proteins, named adipokines. These include leptin, adiponectin, tumor necrosis factor-α, and interleukin (IL)-6. These molecules are not simply bystanders but exert different effects, which could improve or impair metabolic responses. Adipokines have been implicated in both NAFLD and CVD pathogenesis [144]. In particular, leptin and adiponectin may be appropriate biomarkers of NAFLD: low levels of adiponectin may predict independently the development of NAFLD, whereas leptin may be a significant predictor of NAFLD only in subjects with weight gain [145]. In line with this evidence, we previously demonstrated that adiponectin levels are independently associated with the histological severity of NAFLD and the *I148M PNPLA3* genotype may represent a genetic determinant of serum adiponectin [146]. In humans, adiponectin levels are positively correlated with HDL-C and inversely with TG and small-dense atherogenic LDL-C [147,148]. Leptin levels are instead raised in patients with vascular disease, including those

with myocardial infarction and heart failure, as well as in those with coronary artery calcification and higher c-IMT [149].

While obesity, particularly abdominal obesity, is an almost general accompanying marker of NAFLD, ectopic fats (pericardial fat, epicardial fat and pericoronary fat) are also of interest. All these can be measured by computed tomography (CT). Epicardial fat, in particular, is strongly associated with the risk of myocardial infarction [150] and the presence and progression of coronary artery calcification [151]. More recent findings indicate that epicardial fat thickness (EFT) is also strongly associated with obesity linked IR and also with liver steatosis [152]. EFT can be easily determined by echocardiography and is independently associated with left ventricular diastolic dysfunction and atherosclerotic lesions, as assessed by c-IMT. In addition, EFT can provide an early marker of CV injury in patients with IR [153].

The accumulation of ectopic fat can thus be a marker of NAFLD and, while in humans it is best represented by EFT, in rodents it may be characterized by hepatocyte accumulation of neutral lipids forming lipid droplets (LDs) surrounded by proteins of which perilipin2 (PLIN2) is a major component [154]. PLIN2 appears to be a potentially protective factor against NAFLD [155], since reduction of *PLIN2* in monocytes occurs after bariatric surgery [156]. As a general consequence, obese patients with NAFLD treated with bariatric surgery show improved insulin sensitivity and reduced liver and monocyte fat accumulation [6].

4.4 Inflammation. Chronic liver disease, as in the case of NAFLD, is fuelled by hepatic inflammation, being macrophages essential players in controlling this condition. Chronic fat overload induces liver cell death, resulting in the release of danger associated molecular patterns triggering macrophage activation [157]. During chronic liver injury, the increased recruitment of monocyte-derived macrophages and Kupffer cell populations leads to increased circulating levels of systemic inflammatory markers, including IL-1, IL-6 and subfamilies of IL-20 [158]. This process could worsen the CVD risk through endothelial dysfunction, altered vascular tone, raised plaque formation and clotting [159].

4.5 Hypertension. Hypertension is one of the strongest risk factors for almost all different CVDs, including coronary and peripheral disease, left ventricular hypertrophy and valvular heart

diseases, atrial fibrillation, stroke and renal failure [160]. Hypertension is definitely associated with IR and may be contributory or just associated with NAFLD [161]. The similarities between hypertension and NAFLD have led to the evaluation of potential treatments effective in both conditions. Lifestyle modification is an important strategy to manage NAFLD and hypertension. At the moment, however, only weight loss, avoidance of alcohol and some agents reducing IR such as metformin lead to blood pressure reduction [162], thus improving both conditions. Proper strategies should be, however, the object of future investigations.

4.6 Microbiome. The interaction between gut and liver, the so-called "gut-liver axis" plays a critical role in the development and progression of NAFLD in both children and adults. An intact intestinal barrier is able to antagonize the translocation of bacterial products, while allowing active transport of nutrients across tight junctions. As reported in a recent meta-analysis, intestinal permeability appears to be raised in NAFLD patients, being associated with the degree of hepatic steatosis [163]. In this complex scenario, a crucial role is played by the intestinal microbiota, playing a major role in NAFLD. Recent data indicate that microbiome derived metabolites predict fibrosis and cirrhosis in NAFLD [164]. Gut flora and intestinal permeability regulate glucose, lipid and choline metabolism and have a clear impact on intestinal permeability [165]. Increased circulating levels of bacterial products, *i.e.* lipopolysaccharides and other bioactive compounds, lead to the intrahepatic activation of proinflammatory cells and hepatocytes via stimulation of toll-like receptor-2 (TLR-2) [166]. In the attempt to find a link with CVD, one of the best candidates is represented by trimethylamine oxide (TMAO), derived from the metabolism of choline and carnitine by the gut microbiome [167]. TMAO has been extensively reported to be associated with the risk of fatal and non-fatal CV events. Its precursor trimethyllysine predicts near- and long-term CV events in patients with chest pain and acute coronary syndrome [168]. Conversely, although few studies have described changes in circulating levels of TMAO in NAFLD, it seems that TMAO associates with the severity of disease [169] particularly in obese T2D patients with NASH [170].

4.7 Epigenetics, NAFLD and CV risk. In a frame depicting NAFLD as a systemic disorder, miRNAs involved in the regulation of hepatic cholesterol and lipid metabolism, *e.g.*, miR-122, miR-33a/b,

and miR-29, may mediate the development of atherosclerosis and CVD [171]. In apoE$^{-/-}$ mice, miR155 was significantly up-regulated in the aortas, and miR-155 deficiency inhibited the development of atherosclerosis. In miR155$^{-/-}$/apoE$^{-/-}$ double KO mice, exposure to a high fat diet induced obesity, adipocyte hypertrophy and NAFLD [172]. In humans, miR-34 was upregulated in patients with CAD and more so in individuals with NAFLD [172].

Finally, epigenetic changes and alterations in long non-coding RNAs (lnc-RNAs) expression are also involved in CVD. DNA methylation has been associated with atherosclerosis. In apoE$^{-/-}$ mice, DNA methylation precedes any histological sign of atherosclerosis and is correlated with dyslipidemia. Moreover, DNA methylation is associated with inflammatory markers such as IL-6, IL-8, IL1-β, C-reactive protein and vascular cell adhesion molecule-1 [173]. The expression of LncRNAs, known to be relevant in atherosclerosis, in plasma samples of 300 patients with CAD identified H19 and LIPCAR as independent predictors of CV damage [174].

5. Dietary approaches to NAFLD. Dietary intervention is the mainstay approach for the management of NAFLD, with a weight loss of 7-10% roughly considered as the goal. NAFLD patients can benefit by adhering to the Mediterranean low-carbohydrate diet, which can reduce hepatic steatosis and is recommended as the first-line dietary intervention in these patients [175]. Very recently the concept of green Mediterranean diet, enriched with specific green polyphenols, has been tested in the DIRECT PLUS (Dietary Intervention Randomized Controlled Trial Polyphenols Unprocessed) trial. Patients allocated to this approach had a larger intrahepatic fat loss (-2% absolute change) compared to those allocated to the standard Mediterranean diet (-1% absolute change) or a healthy dietary approach (-0.7% absolute change) [176].

Very low energy diet (VLED) intake for periods of 6 days to 12 weeks can reduce liver volume in addition to visceral and subcutaneous adipose tissue and the reduction of liver volume appears to be directly related to a reduction in relative body weight [177]. As shown in an earlier study, a volume reduction of 20% was achieved after 12 weeks of VLED [178]. Interestingly, a significant reduction in liver fat, assessed by non-invasive quantitative magnetic resonance imaging (MRI), was observed after just 3 days of a low carbohydrate diet in normal volunteers. After 10 days, liver fat was reduced in the range between -1 to - 5% in all subjects [179].

In view of the correlation between liver fat and total/animal protein intake, an attempt to reduce hepatic fat was carried out in the GRAANDIOOS (Improving Resilience With Whole Grain Wheat) study in 50 overweight subjects. Patients received either 98 g/d of whole grain wheat or refined wheat and after 12 weeks there was a 49.1% rise of liver fat in the refined wheat group compared to stable levels in the whole grain wheat group [180].

Several dietary components have been investigated for their association with the emergence of NAFLD. Among these are low intakes of PUFA and high intakes of saturated fat and cholesterol [181]. These findings have led to a number of randomized trials in patients with NAFLD by comparing dietary advice alone or with supplementations. In the case of ω-3 PUFA a recent trial randomized 78 patients with NAFLD and hypertriglyceridemia to 4 g ω-3 fatty acids vs 200 mg fenofibrate or placebo for 12 weeks [182]. Regardless of the TG reduction, fenofibrate raised liver volume, whereas ω-3 did not reduce liver fat, with no changes in liver enzymes and reduction of FGF21, independent of *PNPLA3* polymorphism (I148M). This last finding supports the divergent effects of fenofibrate compared with ω-3 on liver lipid accumulation and some potential benefits exerted by ω-3 FA on hepatic metabolism [183]. Since the ω-6 to ω-3 ratio in the Western diet is on the average 15:1, van Name et al [184] investigated in a series of obese youth with NAFLD, the impact of a low ω-6:ω-3 PUFA ratio (4:1) normo-caloric diet on hepatic fat amelioration. The treated youths had a -25.8% reduction of liver fat with no changes in weight as well as a reduction in TG (-21.9%) with a -34% reduction in ALT and in oxidized TG metabolites. These changes were modulated by the *PNPLA3* rs738409 genotype.

Low carbohydrate ketogenic diets have gained popularity in the treatment of obesity, T2D and also NAFLD [185]. This type of approach appears to be promising in the case of liver steatosis, where a hypocaloric ketogenic diet was found to reduce intrahepatic TG in 6 days by up to -45% despite increased circulating FFA levels [186]. The highest reduction occurred in subjects homozygous for the *PNPLA3*-148 MM allele, *i.e.*, - 45 vs + 18% for the PNPLA3-148II group. This study, showing raised FFA in spite of reduction of liver fat, indicates a change of insulin action on liver FFA delivery, suggestive of impaired re-esterification of FA into complex lipids by altered mitochondrial fluxes. Quantification of liver mitochondrial metabolism in NAFLD patients under a ketogenic diet was very recently reported by Luukonen et al [187]. In these patients, isotopic

infusions of the three precursors, glucose, hydroxybutyrate and lactate after 6 days of ketogenic diet reduced intrahepatic TGs by

-31%, again with raised FFA and reduced IR. FFAs appear to be partitioned toward ketogenesis, due to a reduction in serum insulin (-53%) and of the citrate synthase flux. This latter was attributed to a raised mitochondrial redox state (+167%) with a -45% reduction of leptin and -21% of triiodothyronine. These findings may explain the reversal of NAFLD by ketogenic diet with altered hepatic mitochondrial fluxes and redox state promoting liver ketogenesis rather than synthesis of liver TG.

The *PNPLA3* I148M variant represents a first and until now almost unique example of a genetic factor for which a clear interaction between ~~the~~ gene and environment has been robustly demonstrated [188]. At the nutritional level, *PNPLA3* expression is transcriptionally modulated by the activation of the SREBP1c/Liver X Receptor (LXR) pathway induced by hyperinsulinemia and by carbohydrate feeding [189]. In the double-blind placebo controlled WELCOME (Treatment of Non Alcoholic Fatty Liver Disease With n-3 Fatty Acids) trial, 103 adult patients with NAFLD were randomized to receive a supplementation of ω-3 fatty acids (long chain PUFA), including docosahexaenoic (DHA) and eicosapentaenoic acid (EPA) or placebo for 15–18 months. *PNPLA3* homozygous patients displayed an independent association with a decreased percentage of DHA tissue enrichment during the trial but not with changes in serum TG concentrations [190]. Consistently, in another randomized controlled trial, it was tested whether the *PNPLA3* I148M variant is associated with the response to DHA (250 or 500 mg/day) in 60 children with NAFLD for 24 months. This study demonstrated, however, that the 148M allele is associated with no beneficial effects of DHA supplementation on liver fat, showing a doubling of risk of severe steatosis at the end of the trial [191]. These findings were supported by Santoro et al. showing that PNPLA3 is involved in ω-3 FA mobilization in the liver [192]. These may down-modulate SREBP1c expression and the *PNPLA3* I148M variant is associated with lower DNL despite the substantial increase of hepatic fat content, thus explaining the lower response to DHA+EPA therapy in these patients.

5.1. Nutraceuticals. A number of nutraceuticals has been tested for their effects on NAFLD, *e.g.*, silymarin, vitamin E, vitamin D, or resveratrol). Most of these have found support mainly from

anecdotical reports with no clear evidence of benefit. As elsewhere reviewed [193], the most widely used nutraceutical is silymarin, a powerful antioxidant agent extracted from milk thistle (Silybum marianum) with a specific liver tropism. Sylimarin has a low bioavailability, but, when given in combination with vitamin E, exerts a beneficial activity in NAFLD patients. Importantly, as in the case of resveratrol, a long-term appropriate dose supplementation has to be considered in order to achieve a clinical benefit in NAFLD patients [194].

***5.1.2 Probiotics*.** Obesity and nutrition may alter intestinal permeability producing a favorable micro-environment for bacterial overgrowth, mucosal inflammation and translocation of both invasive pathogens and harmful byproducts which influence liver fat composition and exacerbate pro-inflammatory and fibrotic processes [195,196]. Combination of probiotics, *i.e.* non pathogenic microorganisms with health benefits, with prebiotic fibers containing non digestible carbohydrates, modulates specific changes in the composition and activity of gastrointestinal microbiota, leading to the production of active synbiotics [197]. The use of a synbiotic yogurt (10^8 colony-forming unit (CFU) of *Bifidobacterium animalis lactis* as a probiotic and 1.5 g inulin as a prebiotic), has led to a significant reduction of liver enzymes, cholesterol, TGs and the grade of steatosis, in 102 patients with NAFLD and obesity [198]. Microbial-derived metabolite production, in particular short chain fatty acids can finally be important byproducts of prebiotic fibers [199]. In particular, butyrate can reduce inflammatory mediators and its role in NAFLD development is being currently investigated [200].

***6. Drug treatments*.** The numerous reports on dietary approaches for the management of liver steatosis/NAFLD do not match equivalent progress in drug treatment. Although up to now there is not a single drug specifically targeted to NAFLD, roughly 300 molecules are in various stages of development with this indication [201].

Metformin has been thought for many years to exert beneficial effects on NAFLD through mechanisms involving an AMPK dependent improvement of hepatic glucose metabolism and increased uptake into muscle cells [202]. However, this mechanism has been recently challenged [203]. Newer activities of metformin have been detected, in particular at the intestinal level. Reduced intestinal absorption, by way particularly of reduced endotoxin in portal plasma, leads

to improved intestinal barrier function and markedly attenuates NAFLD induced by fat, fructose and cholesterol rich diets [204]. Metformin intake in humans is associated with a decrement in *Bacteroides fragili*s, a bacterial strain mediating barrier function [205]. Improved insulin sensitivity may be linked to an increased microbiomal prevalence of a beneficial bacterial strain, *i.e.*, *Akkermansia muciniphila*, associated with loss of weight and improved liver fat and function in experimental models [206]. In diabetics given metformin, a high relative abundance of *Akkermansia* in intestinal microbiota with a consequent moderate loss of weight was reported [207].

6.1 Agents acting on the PPAR system. While the PPARα activator fenofibrate did not appear to provide any significant benefit on NAFLD [182], because of its potential to raise liver volume and somewhat elevate liver lipid content, a beneficial activity can be exerted by the new agent Pemafibrate. It is >2,000-fold more selective for PPARα vs either PPARγ or PPARδ [208] . Preclinical studies in mice found that pemafibrate improved macrophage accumulation, ballooning degeneration of hepatocytes, and NAFLD without affecting TG accumulation in the liver [209]. This evidence has been confirmed in NAFLD patients in whom pemafibrate improved markers of hepatic inflammation, function and fibrosis [210]. Moving to the CV risk, the PROMINENT (Pemafibrate to Reduce Cardiovascular Outcomes by Reducing Triglycerides in Patients with Diabetes) study will evaluate the efficacy of pemafibrate in reducing major adverse CV events in T2D patients with elevated TG. It is expected to be completed in 2024 [211].

Pioglitazone has been extensively studied in the treatment of NASH [212]. By improving liver insulin sensitivity, pioglitazone can provide CV benefit, particularly in secondary prevention [213]. The earlier PPARγ activator rosiglitazone had been evaluated in patients with NASH in the FLIRT (Rosiglitazone for nonalcoholic steatohepatitis: one-year results of the randomized placebo-controlled Fatty Liver Improvement with Rosiglitazone Therapy) trial. One-year treatment (8 mg/day) led to a mean reduction of 47% in steatosis compared to placebo (-16%) without significant improvement in histological parameters [140]. The initial FLIRT trial was followed by a 2-year extension (FLIRT2 trial) without further significant improvement [214]. More convincing data have been provided by RCTs with pioglitazone, mainly in NASH. In patients with biopsy proven NASH and either prediabetes or T2D, pioglitazone (titrated to 45 mg/day) in adjunction to a hypocaloric diet improved inflammation, ballooning, necrosis and steatosis compared to placebo

[215]. The PIVENS (Pioglitazone vs Vitamin E vs Placebo for Treatment of Non-Diabetic Patients With Nonalcoholic Steatohepatitis) trial, comparing pioglitazone (30 mg/day) vs vitamin E (800 IU/day) or placebo gave evidence that more patients on vitamin E achieved the primary composite endpoint, including improvement in the NASH activity score, while the difference between pioglitazone and placebo did not reach the prespecified goal. Although pioglitazione did not reduce fibrosis, the histological resolution of NASH was found in 47% of patients compared to 21% on placebo (p=0.001) [216]. The side effects of pioglitazone remain of concern, since it may cause weight gain, sodium/water retention and osteoporosis and potentially an increased risk of CV events [217].

6.2 Newer antidiabetic agents. The sodium/glucose cotransporter-2 (SGLT2) inhibitors have provided a novel approach to diabetes. SGLT2 are expressed in the proximal renal tubules and are responsible for the majority of glucose reabsorption. SGLT2 inhibitors promote urinary glucose excretion and, in addition, reduce body weight and blood pressure with low risk of hypoglycemia [218]. These compounds appear to have an excellent profile for managing steatosis, as shown in animal models where the drugs reduced both liver TG accumulation and fibrosis [219]. After positive data in animal models [220], in humans, treatment with SGLT2 inhibitors was shown to improve liver structure and function in patients with T2D [221]. SGLT-2 inhibitors reduce CV deaths and all-cause deaths compared to placebo (OR 0.77, 95%CI 0.60-0.98 and OR 0.67, 95%CI 0.54-0.84) [222].

Two new classes of antidiabetic agents have been tested in NAFLD. Agents acting on the glucagon-like peptide 1 (GLP-1) are further divided into GLP-1 receptor (GLP-1R) agonists and dipeptidylpeptidase-4 (DPP-4) inhibitors. GLP-1 is a naturally occurring gastrointestinal (GI) hormone secreted by L-cells of the distal small intestine, that regulate glucose in systemic and splanchnic vessels by stimulating glucose-dependent insulin secretion and inhibiting glucagon release [223]. GLP-1R agonists such as liraglutide, exenatide, dulaglutide and senaglutide seem to have a potential protective action on liver function. Seventy-two week treatment with semaglutide (0.4 mg) resulted in a significantly higher percentage of patients with NASH resolution than placebo, 43% vs 33%, respectively [224].

Agents acting by a similar mechanism as the GLP-1R agonists are the dideptidylpeptidase-4 (DPP-4) inhibitors. DPP-4 is an enzyme breaking down bioactive enzymes, including the glucose dependent insulinotropic polypeptide (GIP) [225]. Inhibition reduces insulin secretion and suppresses glucagon. DPP-4 inhibitors were developed after the identification of the therapeutic activity of GLP1-R, in order to delay rapid inactivation and increase the incretin effect. The activity on NAFLD of DPP-4 inhibitors (sitagliptin, saxagliptin, linagliptin, alogliptin and vidagliptin) seems to be due to suppressed proinflammatory and profibrotic phenotypes of macrophages [226]. Although retrospective studies indicated reduced lipid abnormalities in patients with NASH or T2D, as well as improvement of liver enzymes [227], more recent trials with sitagliptin did not provide clear statistically significant effects [228].

6.3 Farnesoid X receptor (FXR) agonist. FXR is a bile acid nuclear receptor playing a diversified role in lipoprotein and glucose metabolism, and also controlling hepatic fibrosis and inflammation [229]. A reduced expression of FXR raises the risk of developing NASH and HCC in mice [230], a finding confirmed by the decreased FXR expression in patients with NAFLD [231]. The novel opening on the management of FXR deficiency or reduced expression was provided by obeticholic acid (OCA), a semisynthetic variant of the natural bile acid chenodeoxycholic acid [232]. OCA is approved for the treatment of primary biliary cholangitis and progressive autoimmune liver disease in patients with inadequate response to, or unable to tolerate, ursodeoxycholic acid [233]. In NAFLD patients with T2D, OCA (25 mg/day) raised insulin sensitivity by 28% and by 20.1% with the 50 mg/day dose. A significant reduction in markers of liver fibrosis with a lower dose was also found [232].

In the FLINT (Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis) trial, enrolling 283 biopsy-proven NAFLD patients, 72-week treatment with OCA (25 mg/day) led to a histological improvement in 45.4% of patients vs 21.1% in the placebo group [234]. More recently, Younossi et al [235] in the REGENERATE (Randomized Global Phase 3 Study to Evaluate the Impact on NASH With Fibrosis of Obeticholic Acid Treatment) study reported improvement in fibrosis and other key components of NASH upon 18 months (interim analysis) of treatment with OCA (clinical outcomes are expected). FXR agonism can lead to acute liver injury in rodent models of cholestasis. These findings could perhaps improve

understanding of side effects of OCA, *i.e.*, itching and hepatic decompensation in cirrhotic patients. However, acute liver injury has never been reported in the clinic [236].

6.4 Ezetimibe. Ezetimibe, widely used for the treatment of hypercholesterolemia and CV risk reduction [237] may be effective in treating NAFLD, especially in combination with fish oil [238]. Clinical studies with ezetimibe (without fish oil) have provided discordant results [239]. Ezetimibe inhibits the Nieman-Pick C1-Like 1 (NPC1L1) sterol receptor, a cellular protein expressed in enterocytes and in the liver. It regulates dietary and biliary cholesterol absorption [240]. A stimulated expression of cholesterol efflux transporters was very recently described in rats on fish oil. Those receiving 10% fish oil + 0.005% ezetimibe showed no increase in fecal cholesterol but a dramatic rise in the expression of *ATP-binding cassette (ABC)G5/ABCG8* gene expression in the liver. Raised gene expression was associated with an 84% and 86% reduction of hepatic TG and cholesterol, respectively. ABCG5/ABCG8 form a heterodimeric complex responsible for biliary and transintestinal secretion of cholesterol and dietary sterols [241]. Finally, the recent finding that the overexpression of NPC1L1 in the liver exacerbates NAFLD, a pathological finding rescued by ezetimibe, could be the leverage to study patients with higher levels of NPC1L1 [242].

7. Conclusions. Striking evidence from epidemiological and observational studies gives an indisputable link between NAFLD and a raised risk of CVD, whereas mixed results were reported in carriers of allelic variants causally related to NAFLD development (*e.g.*, *PNPLA3*, *TM6SF2*, *MBOAT7*, *GCKR* and *17β-HSD13*). However, these associations should be reconsidered following the proposal to change diagnostic criteria, in order to identify MAFLD patients with fatty liver disease who are at high risk of disease progression or have a greater risk of CVD. Indeed, besides embracing metabolic abnormalities, MAFLD is also comprehensive of inflammatory markers, *i.e.*, hsCRP $\geq$ 2 mg/L, a well-known CVD risk factor [243]. Finally, in the present lack of a targeted pharmacological approach, the MAFLD definition narrowing this disease to a metabolic disorder could encourage drug repurposing, *e.g.*, SGLT2 inhibitors or new selective PPARα agonists.

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Table 1. Dominant genetic modifiers known to shape both NAFLD susceptibility and progression.

Gene	Liver cell type	Function	CAD risk
PNPLA3	- Hepatocytes - Stellate cells	- Lipid droplet remodeling - Modulation of retinol production and release	- reduced OR: 0.92 (95%CI 0.87-0.97)
TM6SF2	Hepatocytes	- $VLDL_1$-TG production was 35% lower in homozygous *TM6SF2 E167K* carriers	- reduced OR: 0.78 (95%CI 0.65-0.93)
MBOAT7	- Hepatocytes - Hepatic sinusoidal cells - Stellate cells	- Remodeling of phosphatidylinositol	- neutral effect (OR: 1.01 (0.37-1.05) - raised risk of venous thromboembolism
HSD17B13	- Hepatocytes	- Lipid droplet remodeling - Retinol metabolism	n.a.
GCKR	- Hepatocytes	- Raised glycolytic flux - Regulation of *de novo* lipogenesis	n.a.

HSD17B13, Hydroxysteroid 17-Beta Dehydrogenase 13; *MBOAT7*, Membrane Bound O-acyltransferase Domain-containing 7; NAFLD, nonalcoholic fatty liver disease; *PNPLA3*, Patatin-like Phospholipase Domain–Containing 3; *TM6SF2*, Transmembrane 6 Superfamily Member 2 gene. Adapted with permission of Elsevier [244].

Figure legend

Figure 1. *Proposed criteria for the clinical diagnosis of MAFLD* (with permission of Elsevier [14]).

Figure 2. *Contribution of NAFLD to the develpoment of cardiovascular disease*. The mechanisms underlying NAFLD pathogenesis are multifactorial. Parallel hits as environment, gut-microbiota, genetics and epigenetics participate to the disease onset and progression. The first step in NAFLD development is hepatic fat infiltration, mainly due to insulin resistance, which leads to adipose tissue lipolysis with the consequent efflux of free fatty acids to the liver. In addition, the compensatory hyperinsulinemia activates the *de novo* lipogenesis, trough SREBP1c, and exacerbates fat accumulation. The adipose tissue releases adipokines, such as leptin, adiponectin, TNF-α and IL-6. Excess fat is cleared from the liver by an enhanced lipoprotein secretion and mitochondrial β-oxidation. The latter results in increased reactive-oxigen species production leading to the activation of inflammatory pathways. The endothelial dysfunction and lipid accumulation in arteries is linked to inflammation, which results in lipid accumulation in macrophages that are called "foam cells". This leads to alteration of intima-media thickness and plaques formation. IL, interleukin; Hydroxysteroid 17-Beta Dehydrogenase 13 (*HSD17B13*); Membrane Bound O-acyltransferase Domain-containing 7 (*MBOAT7*); NAFLD, nonalcoholic fatty liver disease; NASH, Nonalcoholic steatohepatitis; TNF, tumor necrosis factor; Patatin-like Phospholipase Domain–Containing 3 (*PNPLA3*), SREBP1c, sterol regulatory element-binding protein; Transmembrane 6 Superfamily Member 2 gene (*TM6SF2*).

Figure 3. *Impact of genetic risk variants on liver damage.* Comparison of the impact of risk variants *PNPLA3* I148M (rs738409), *TM6SF2* E167K (rs58542926), *GCKR* P446L (rs1260326) and *MBOAT7* rs641738 on hepatic fat vs. liver damage. Panel a) histological steatosis vs. ballooning; panel b) histological steatosis vs. necroinflammation; panel c) histological steatosis vs. fibrosis; panel d) hepatic fat content vs. serum ALT levels. Reproduced with permission of Wiley [23].

Figure 4. *Pharmalological approaches which reduce CV risk and may potentially be used in NAFLD patients*. Metformin inhibits AMPK pathway; Pemafibrate activates PPARα and Pioglitazone

activates PPARγ; Obeticholic acid inhibites FXR; Liraglutide, exenatide, dulaglutide and senaglutide are GLP-1R agonist; Sitagliptin, saxagliptin, linagliptin, alogliptin, and vidagliptin are DPP-4 inhibitors; Glifozines are SGLT-2 inhibitors. CV, cardiovascular; GLP-1R, glucagon-like peptide 1; DDP-4, dideptidylpeptidase-4. NAFLD, nonalcoholic fatty liver disease; CVD, cardiovascular disease.

Figure 1

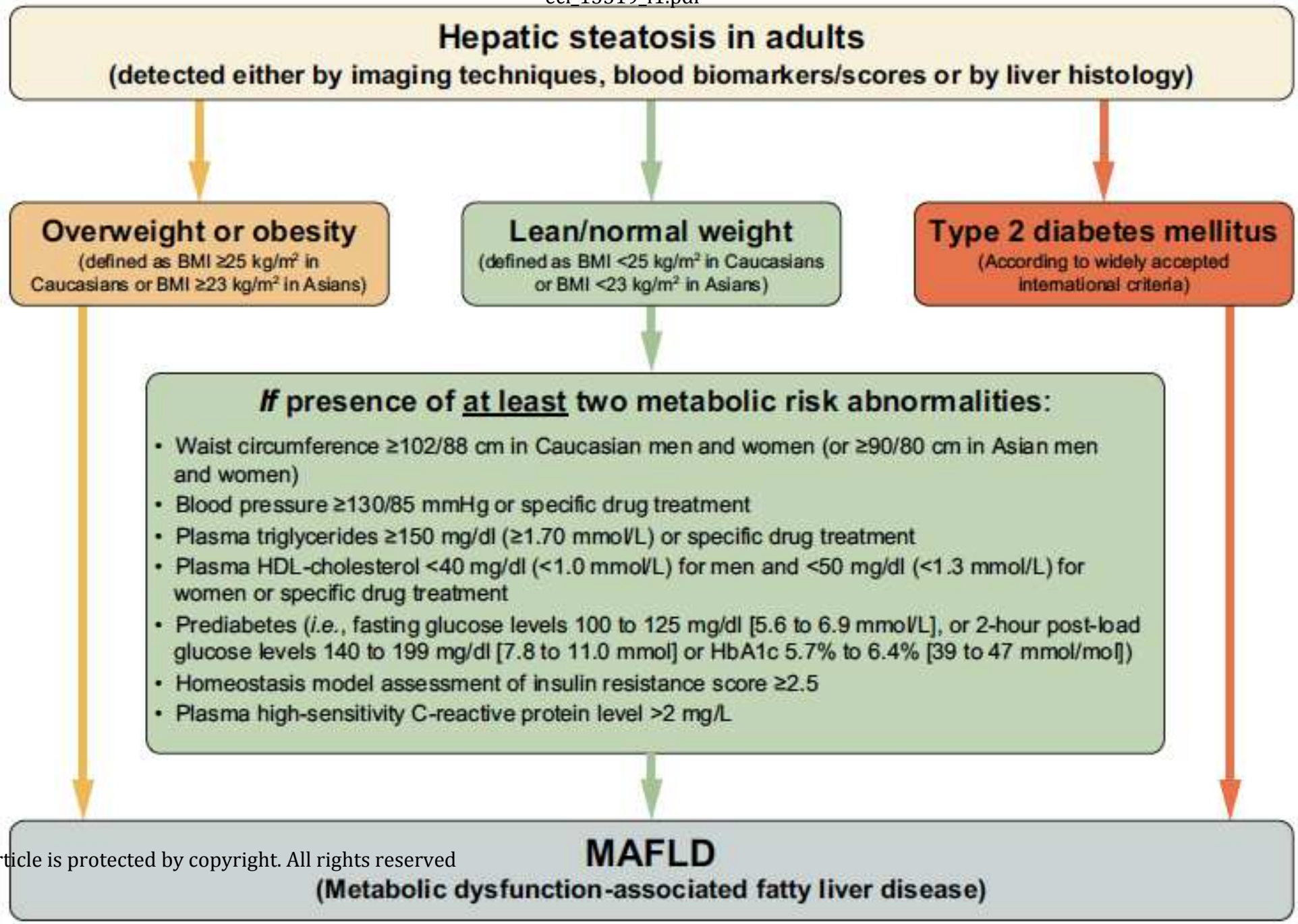


Figure 2

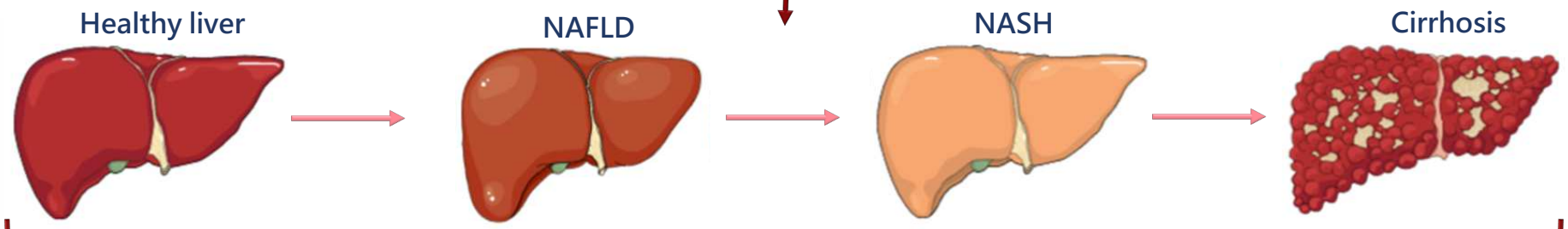
Environment
Age
Gender
Obesity
Diabetes

Gut Microbiota
Leaky gut
Dysbiosis
Inflammation

Genetics
PNPLA3
TM6SF2
MBOAT7
GCKR
17β-HSD13

Epigenetics
miRNAs
DNA methylation
Histone modifications

NAFLD progression



Atherogenic triggers

- Insulin Resistance
- Dyslipidemia
- Oxidative stress
- Endotelial dysfunction
- Lipid accumulation

- Cytokine release
- Leptin
- Adiponectin
- TNF-α
- IL-6

- Macrophage activation
- Cytokine release
- IL-1
- IL-6
- IL-20

CVD progression

Hypertension and Inflammation:
- endothelial dysfunction
- altered vascular tone
- raised plaque formation and clotting

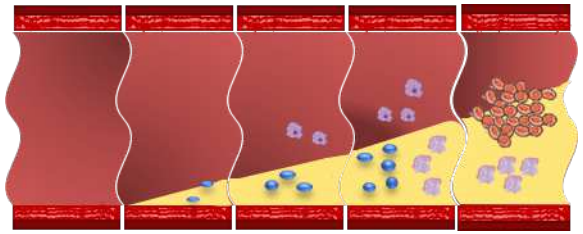


Figure 3

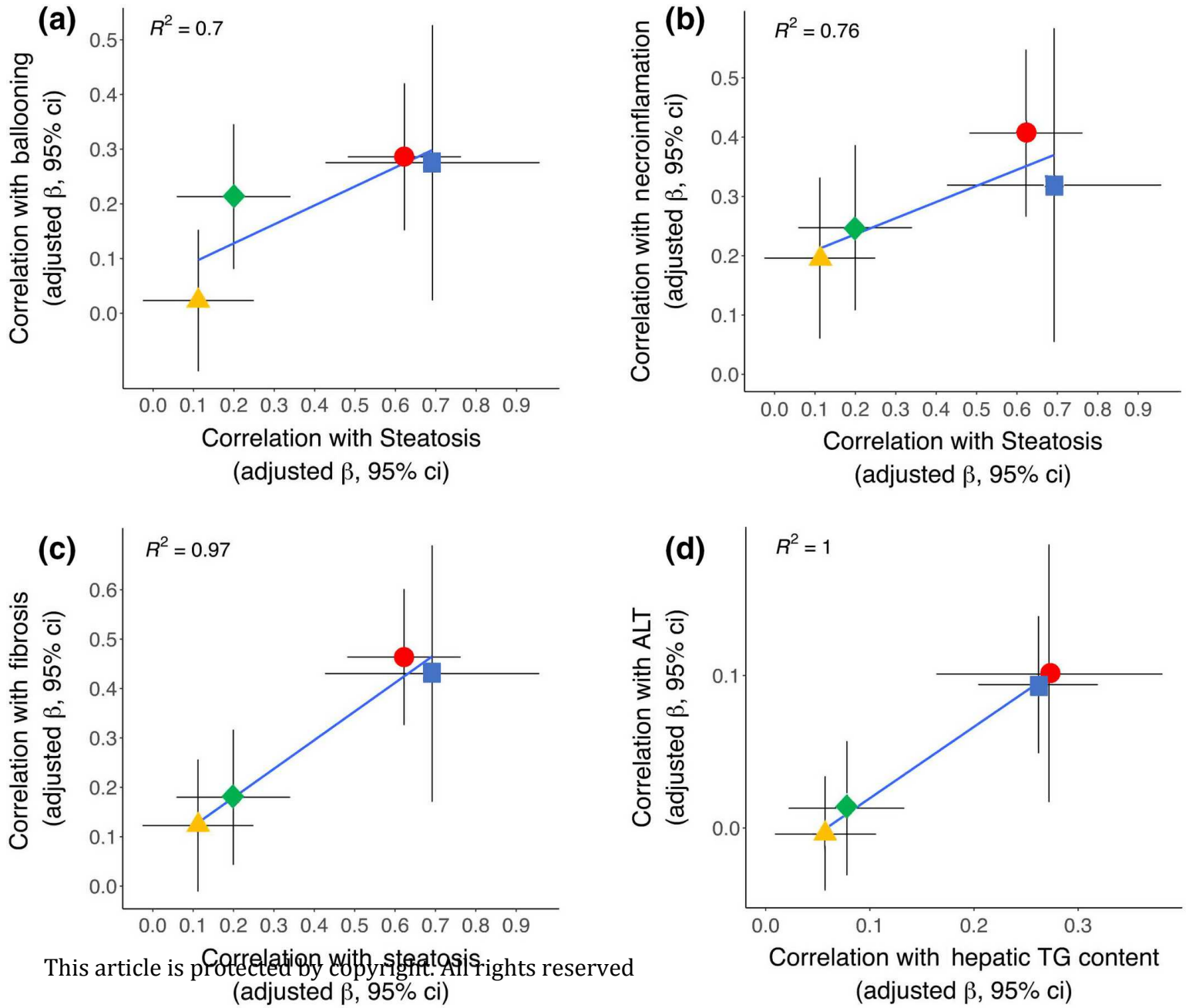


Figure 4