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## Promoting genetics in non-alcoholic fatty liver disease: Combined risk score through polymorphisms and clinical variables

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### Abstract

Non-alcoholic fatty liver disease (NAFLD) has a prevalence of approximately 30% in western countries, and is emerging as the first cause of liver cirrhosis and hepatocellular carcinoma (HCC). Therefore, risk stratification emerges as fundamental in order to optimize human and economic resources, and genetics displays intrinsic characteristics suitable to fulfill this task. According to the available data, heritability estimates for hepatic fat content range from 20% to 70%, and an almost 80% of shared heritability has been found between hepatic fat content and fibrosis. The rs738409 single nucleotide polymorphism (SNP) in patatin-like phospholipase domain-containing protein 3 gene and the rs58542926 SNP in transmembrane 6 superfamily member 2 gene have been robustly associated with NAFLD and with its progression, but promising results have been obtained with many other SNPs. Moreover, there has been proof of the additive role of the different SNPs in determining liver damage, and there have been preliminary experiences in which risk scores created through a few genetic variants, alone or in combination with clinical variables, were associated with a strongly potentiated risk of NAFLD, non-alcoholic steatohepatitis (NASH), NASH fibrosis or NAFLD-HCC. However, to date, clinical translation of genetics in the field of NAFLD has been poor or absent. Fortunately, the research we have done seems to have placed us on the right path: We should rely on longitudinal rather than on cross-sectional studies; we should focus on relevant outcomes rather than on simple liver fat accumulation; and we should put together the genetic and clinical information. The hope is that combined genetic/clinical scores, derived from longitudinal studies and built

on a few strong genetic variants and relevant clinical variables, will reach a significant predictive power, such as to have clinical utility for risk stratification at the single patient level and even to estimate the impact of intervention on the risk of disease-related outcomes. Well-structured future studies would demonstrate if this vision can become a reality.

**Key words:** Non-alcoholic fatty liver disease; Single nucleotide polymorphism; Patatin-like phospholipase domain-containing protein 3; Transmembrane 6 superfamily member 2; Membrane bound O-acyltransferase domain containing 7; Glucokinase regulatory gene; Risk score; Non-alcoholic steatohepatitis; Non-alcoholic steatohepatitis cirrhosis; Hepatocellular carcinoma

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**Core tip:** Risk stratification is emerging as fundamental in the non-alcoholic fatty liver disease (NAFLD) epidemic. Due to the strong heritability of hepatic fat content and of liver fibrosis progression, genetics is perfectly suitable to fulfill this task. However, notwithstanding the fact that different gene variants have been robustly associated with NAFLD and with its evolution, translation to the clinical ground has been poor or absent. The evidence produced suggests that combined risk scores, created by integrating different genetic and clinical information and tested with respect to relevant outcomes in longitudinal studies, may represent the way for genetics to gain strength in NAFLD diagnostics.

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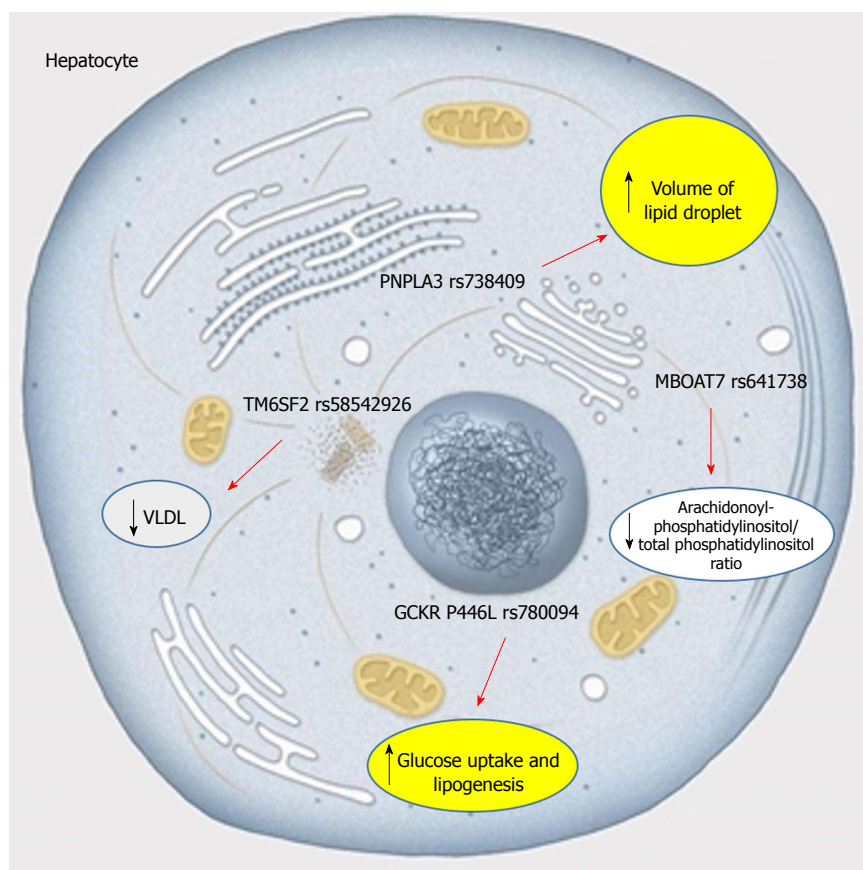
## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has a prevalence of 25%-30% in the general population and has become the main reason for referrals to hepatology services<sup>[1]</sup>. While the majority of NAFLD patients have simple steatosis or steatosis with non-specific inflammation [non-alcoholic fatty liver (NAFL)], and will never evolve to advanced liver disease, a little subgroup of them develops non-alcoholic steatohepatitis (NASH) and has the potential to progress until liver cirrhosis and hepatocellular carcinoma (HCC). Since even a little slice of a big cake is quite a big slice, NAFLD is already becoming the leading cause of advanced liver disease in western countries. In the fight against this alarming epidemic, risk stratification emerges as fundamental. Who among NAFLD patients deserves specific hepatological follow-up

and interventions, and who could simply receive lifestyle indications by a family physician or, eventually, would be better left to the care of diabetologist, cardiologist or nephrologist, if necessary? Answering this question means optimizing human and economic resources.

The diagnosis of NASH vs NAFL has been classically considered crucial in the prognostic assessment of NAFLD, since patients with NASH have demonstrated a reduced global or, at least, liver-related survival with respect to those with NAFL<sup>[2,3]</sup>. However, the multiple noninvasive scores proposed, based on clinical and/or non-routine biochemical variables<sup>[4]</sup>, have never reached a sufficient diagnostic accuracy to be proposed for clinical practice, and liver biopsy is still recommended for a definite NASH diagnosis<sup>[1,5]</sup>. Moreover, recent evidence has questioned the discriminatory potential of a diagnosis of NASH, since also patients with NAFL have shown to deposit a certain degree of fibrosis, and a subgroup of them are even classifiable as rapid fibrosis progressors<sup>[6]</sup>. Consequently, as recommended in recent guidelines<sup>[1,5]</sup>, it is better to test NAFLD patients for advanced fibrosis, which is the most important determinant of their outcome<sup>[7,8]</sup>. The NAFLD fibrosis score (NFS) and the fibrosis-4 (FIB-4) score are well-validated and simple non-invasive tests that have a high negative likelihood ratio for the exclusion of advanced fibrosis<sup>[9,10]</sup>, and they have been proposed as the first step since patients at low risk for advanced fibrosis according to these scores can be managed in primary care. Conversely, patients at indeterminate or high risk are better referred to secondary care, where liver stiffness measurement with FibroScan (even better if with the XL probe), acoustic radiation force impulse or supersonic shear imaging can further refine the noninvasive assessment of their fibrosis stage<sup>[11,12]</sup>. However, relying on the absence/presence of advanced fibrosis to assess the prognosis of an individual patient presents two clear limitations: first, it seems useful for "secondary" rather than for "primary" prevention; second, and consistent with the first point, it's not useful for a correct classification of the young NAFLD patient.

Genetics displays intrinsic characteristics with the potential to solve these limitations, and, interestingly in this line, a body of recent evidence indicates strong heritability of hepatic fat content<sup>[13]</sup>. Indeed, the role of genetic variation in NAFLD, specifically single nucleotide polymorphism (SNP), has been the focus of extensive research. According to the available data, the heritability estimates range from 20% to 70%, depending on the study design, ethnicity, and the methodology applied for the diagnosis<sup>[14]</sup>. In a recent study on a cohort of well-characterized twins in which steatosis was assessed by magnetic resonance imaging proton density fat fraction (MRI-PDFF), and fibrosis by MRI-elastography, the heritability of steatosis was 0.52 and that of fibrosis 0.5<sup>[15]</sup>. More importantly, steatosis and fibrosis were strongly correlated, and steatosis shared 78% of its heritable content with fibrosis, suggesting that the same genetically-determined mechanisms inducing



**Figure 1 Genetic polymorphisms more consistently associated with non-alcoholic fatty liver disease and their suggested physiopathological role.** Patatin-like phospholipase domain-containing protein 3 is a lipase involved in the hydrolysis of triacylglycerol and is localized in the endoplasmic reticulum and directly on the surface of lipid droplets. Patients with the I148M variant have a reduced enzymatic activity which impairs the mobilization of fatty acids and favors the development of steatosis. Transmembrane 6 superfamily member 2 is involved in the enrichment of triglycerides to apolipoprotein B100 in the pathway of very low density lipoprotein (VLDL) secretion from hepatocytes. The E167K variant causes a retention of triglycerides in hepatic lipid droplets and a reduced secretion of VLDL. A lower expression of membrane bound O-acyltransferase domain containing 7 associated with the rs641738 allele determines changes in the hepatic phosphatidylinositol acyl-chain remodeling, increased hepatic fat content and histological damage. Finally, glucokinase regulatory gene regulates *de novo* lipogenesis by controlling the influx of glucose in hepatocytes. The P446L variant increases hepatic fat accumulation by stimulating lipogenesis and glucose uptake. PNPLA3: Patatin-like phospholipase domain-containing protein 3; TM6SF2: Transmembrane 6 superfamily member 2; VLDL: Very low density lipoprotein; MBOAT7: Membrane bound O-acyltransferase domain containing 7; GCKR: Glucokinase regulatory gene.

disease development may favor its progression in terms of fibrosis. Notwithstanding that, the only concrete translation to the clinic obtained to date consists in the recommendation from the last NAFLD European guidelines to genotype for the two major genetic components of NAFLD identified so far, *i.e.*, the patatin-like phospholipase domain-containing protein 3 (PNPLA3) rs738409 and the transmembrane 6 superfamily member 2 (TM6SF2) rs58542926 polymorphisms, in selected NAFLD patients, but not routinely<sup>[5]</sup>. It's pretty clear that we need to find the way to go further.

## GENETIC VARIANTS MOST CONSISTENTLY ASSOCIATED WITH NAFLD DEVELOPMENT AND SEVERITY

A great amount of evidence on the role of genetics in NAFLD/NASH has been produced during the last 10-15 years by candidate gene and, mainly, genome-wide association studies (GWAS). Many polymorphisms have

been proposed, a few of which have already acquired a recognized role in the physiopathology of NAFLD (Figure 1). Romeo *et al.*<sup>[16]</sup> were the first to report that the rs738409 C>G single nucleotide polymorphism (SNP) in PNPLA3 gene, encoding the isoleucine to methionine variant at protein position 148 (I148M), was strongly associated with increased liver fat content. Subsequently, the I148M variant has been associated with the degree of liver injury and all the histopathological aspects of NAFLD, including the presence of NASH, the degree of fibrosis, evolution to cirrhosis and development of HCC<sup>[17-20]</sup>. Actually, the PNPLA3 polymorphism should be regarded as the strongest determinant of interindividual and ethnicity-related differences in hepatic fat content. The mechanisms through which the PNPLA3 variant contributes to NAFLD development and severity have been extensively investigated, and the latest evidences suggest that the 148M substitution induces a loss of function of PNPLA3 hydrolase activity towards triglycerides and retinyl esters, with their subsequent accumulation in lipid droplets of hepatocytes and hepatic



stellate cells<sup>[21,22]</sup>.

The rs58542926 polymorphism in TM6SF2 has clearly emerged as the second genetic determinant of NAFLD in terms of importance<sup>[23]</sup>. The variant, encoding for an E to K substitution at position 167 resulting in a loss of function, has been associated with a reduced hepatic capability to secrete very low-density lipoprotein and, therefore, with hepatic steatosis and necroinflammation<sup>[24]</sup>. It is not surprising that carriers of the TM6SF2 E167K variant are more susceptible to NASH but are protected against cardiovascular disease<sup>[24]</sup>. Concerning the association between the TM6SF2 rs58542926 polymorphism and liver fibrosis, results have been much more heterogeneous than those obtained for the PNPLA3 variant. In a study of 349 biopsy-proven patients, the TM6SF2 E167K variant was associated with a greater risk of NAFLD-related liver fibrosis<sup>[25]</sup>, and the association with advanced fibrosis was confirmed by Dongiovanni *et al.*<sup>[24]</sup>, even if in this study it was not independent from the presence of NASH. However, other Authors have not replicated this finding, possibly also due to the low frequency of the TM6SF2 E167K variant in their study populations<sup>[26-27]</sup>. Due to the poorness of specific studies, there is no conclusive data regarding the association between the TM6SF2 rs58542926 polymorphism and HCC. Notably, Liu *et al.*<sup>[25]</sup> reported that the TM6SF2 E167K variant confers increased predisposition to NAFLD-related HCC, although the association was not independent from classical risk factors, including gender, age, presence of type-2 diabetes mellitus (T2DM) and cirrhosis. More recently, the TM6SF2 polymorphism was characterized among the independent predictors of NAFLD-HCC even after adjustment for age, sex, T2DM and advanced fibrosis<sup>[28]</sup>.

Another locus in which variation has been robustly associated with NAFLD is that of the glucokinase regulatory gene (GCKR). Indeed, in a metanalysis of 5 previous studies, the rs780094 polymorphism at the GCKR locus has been associated with an approximately 1.2 increased risk of NAFLD<sup>[29]</sup>. Notably, in a large cohort of Italian patients with NAFLD, the rs780094 C>T SNP has been associated also with the severity of liver fibrosis and with high triglyceride levels<sup>[30]</sup>, while studies on the association with HCC are missing. Actually, it seems that the rs780094 polymorphism may be acting as a proxy for a nearby tightly linked SNP, rs1260326, which encodes a common missense GCKR variant. This loss-of-function GCKR mutation reduces GCKR ability to regulate glucokinase in response to fructose-6-phosphate, activating hepatic glucose uptake, increasing the production of malonyl-CoA, and finally favoring lipogenesis<sup>[30,31]</sup>.

Another important polymorphism is the rs641738 variant of the membrane bound O-acyltransferase domain containing 7 (MBOAT7) gene, which was shown to predispose to development and progression of NAFLD in individuals of European descent<sup>[32,33]</sup>. MBOAT7 is involved in phosphatidylinositol remodeling and the

variant was associated with lower protein expression in the liver and changes in plasma phosphatidylinositol species, consistent with decreased MBOAT7 function, determining increased hepatic fat content, more severe liver damage and increased risk of fibrosis<sup>[33]</sup>. Notably, in NAFLD patients, the MBOAT7 rs641738 polymorphism has been associated with HCC, particularly in non-cirrhotic individuals<sup>[28]</sup>.

Very recently, the rs72613567 variant in hydroxysteroid 17-beta dehydrogenase 13 (HSD17B13), an uncharacterized member of the hydroxysteroid 17-beta dehydrogenase family, has been associated with decreased levels of alanine aminotransferase (ALT), and with a reduced risk of NASH and fibrosis, suggesting that this variant allele protects against progression to more clinically advanced stages of chronic liver disease<sup>[34]</sup>. Notably, homozygosity for the polymorphism determined an almost 50% reduction of the risk of nonalcoholic cirrhosis, and the variant was also associated with lower odds of HCC<sup>[34]</sup>. The data just obtained is consistent with HSD17B13rs72613567 altering mRNA splicing, with the synthesis of a truncated protein in human liver<sup>[35]</sup>. Actually, HSD17B13 has enzymatic activity against several bioactive lipid species (*e.g.*, leukotriene B4), which may play a role in lipid-mediated inflammation<sup>[35]</sup>.

Finally, there is one polymorphism deserving attention in the context of NAFLD genetics even if the reference gene is not involved in metabolic processes. The interferon (IFN)- $\lambda$ 3/IFN- $\lambda$ 4 regions participates in the regulation of innate immunity<sup>[36]</sup>. The rs12979860 polymorphism in the IFN- $\lambda$ 3 gene, determining increased IFN- $\lambda$ 3 production, has been associated with increased hepatic inflammation and fibrosis in NAFLD patients, particularly in non-obese ones<sup>[37]</sup>.

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## PURE GENETICS OR COMBINED (GENETIC/CLINICAL) RISK SCORES IN THE ASSESSMENT OF NAFLD PATIENTS: THE EVIDENCE COLLECTED TO DATE

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A number of studies has been produced in which genetic variants, alone (Table 1) or in combination with clinical variables (Table 2), have been tested for their capability to predict the presence of NAFLD, NASH, NASH fibrosis or NAFLD-HCC.

Proof of concept of the additive role of polymorphisms associated with NAFLD in determining liver damage is the direct correlation between the number of variants possessed by single patients and the level of liver enzymes<sup>[38,39]</sup>. Indeed, by evaluating the PNPLA3 rs738409, TM6SF2 rs58542926 and MBOAT7 rs641738 polymorphisms in a population of 320 NAFLD patients, a significant increase of serum aspartate aminotransferase (AST) activity, and a trend for increased ALT and  $\gamma$ -glutamyl transferase (GGT) levels, were observed with the increment of risk alleles<sup>[38]</sup>. Moreover, a polygenic



**Table 1** Overview of the main studies testing pure genetics risk score in the assessment of non-alcoholic fatty liver disease patients

Ref.	Variables included	Risk score formula	Outcomes
Petta <i>et al</i> <sup>[33]</sup> , 2012	IL28B rs12979860 CC and PNPLA3 rs738409 GG	IL28B rs12979860 CC + PNPLA3 rs738409 GG	Higher prevalence of moderate-severe lobular inflammation ( $P < 0.001$ ) and NASH $> 5$ ( $P = 0.02$ )
Nobili <i>et al</i> <sup>[42]</sup> , 2014	PNPLA3 rs738409 C>G, SOD2 rs4880 C>T, KLF6 rs3750861 G>A and LPIN1 rs13412852 C>T	$1/[1+e^{-(0.804 \cdot \text{PNPLA3 GG} \times 1.923 + \text{SOD2 TT} \times 0.564 + \text{LPIN1 TT} \times 0.551 - \text{KLF6 AG-AA} \times 0.324)}]$	AUROC 0.75 (CI: 0.67-0.82, $P < 0.0001$ ) per NASH, cutoff 0.42%-90% sensitivity/36% specificity for NASH
Leon-Mimila <i>et al</i> <sup>[43]</sup> , 2015	PNPLA3 rs738409, LYPLAL1 rs12137855, GCKR rs1260326 and PPP1R3B rs4240624	GRS = Sum of at-risk alleles: 0 for homozygous for the non-risk allele, 1 for heterozygous and 2 for homozygous for the risk allele	GRS $\geq 6$ increased risk of NAS (OR = 2.55, $P = 0.045$ ) compared to those with GRS $\leq 5$
Wang <i>et al</i> <sup>[40]</sup> , 2016	PNPLA3 rs738409 and TM6SF2 rs58542926	Sum of at-risk alleles: 0, 1 or 2 according to the number of minor allele	OR for NAFLD increase of 1.52 per additional risk allele
Di Costanzo <i>et al</i> <sup>[54]</sup> , 2017	PNPLA3 rs738409, GCKR rs1260326 and TM6SF2 rs58542926	Weighted sum of at-risk alleles	A 3 SNPs weighted genetic score $> 0.32$ : five-fold increased risk of NAFLD
Krawczyk <i>et al</i> <sup>[38]</sup> , 2017	PNPLA3 rs738409, TM6SF2 rs58542926 and MBOAT7 rs641738	Sum of at-risk alleles	Association with increasing hepatic fibrosis and steatosis; increase of serum AST activities ( $P < 0.0001$ ), trends for increased ALT ( $P = 0.08$ ) and GGT ( $P = 0.07$ )
Larrieta-Carrasco <i>et al</i> <sup>[39]</sup> , 2018	PNPLA3 rs738409, PNPLA3 rs3810622, SAMM50 rs2143571, ADPOQ rs17366743, COL13A1 rs7101190 and COL13A1 rs12277556	Polygenic risk score = Sum of at-risk alleles 0 for homozygous for the non-risk allele, 1 for heterozygous and 2 for homozygous for the risk allele	9-12 vs 1-4 risk alleles: 65.8% and 48.5% higher ALT and AST levels
Vespasiani-Gentilucci <i>et al</i> <sup>[45]</sup> , 2018	PNPLA3 rs738409, TM6SF2 rs58542926, KLF6 rs3750861	GRS: Sum of at-risk alleles PNPLA3 CC = 0, CG = 1, GG = 2; TM6SF2 CC = 0, CT and TT = 1; KLF6 CC = 0, CT and TT = 1	In healthy subjects: GRS 1-2 vs 0 increases 4-fold and GRS 3-4 vs 0 increases 20-fold the risk of non-cirrhotic NAFLD; in non-cirrhotic NAFLD: GRS 3-4 vs 0 increases 4-fold the risk of NASH cirrhosis
Di Costanzo <i>et al</i> <sup>[41]</sup> , 2018	PNPLA3 rs738409, GCKR rs1260326, TM6SF2 rs58542926 and MBOAT7 rs641738	GRS: Sum of at-risk alleles (0-2); wGRS: Sum of at-risk alleles Beta coefficient	GRS 3 vs 2 higher in NAFLD ( $P = 0.001$ ) wGRS $> 0.28$ : three fold increased risk of NAFLD
Koo <i>et al</i> <sup>[44]</sup> , 2018	PNPLA3 rs738409 and TM6SF2 rs58542926	Sum of at-risk alleles: PNPLA3 CC = 0, CG = 1, GG = 2 TM6SF2 CC = 0, CT and TT = 1	Prevalence of NASH in NAFLD patients: 28.2% (0 allele), 41.8% (1 allele), 63.7% (2 alleles), 69.2% (3 alleles); risk of NASH: 2.04 OR per risk allele

GRS: Genetic risk score; wGRS: Weighted GRS; PNPLA3: Patatin-like phospholipase domain-containing protein 3; SOD2: Superoxide dismutase 2; KLF6: Krueppel-like factor 6; LPIN1: Lipin1; AUROC: Area under the receiver operating characteristic curve; CI: Confidence interval; NASH: Non-alcoholic steatohepatitis; GCKR: Glucokinase regulatory gene; TM6SF2: Transmembrane 6 superfamily member 2; ALT: Alanine aminotransferase; GGT:  $\gamma$ -glutamyl transferase; NAFLD: Non-alcoholic fatty liver disease; AST: Aspartate aminotransferase; MBOAT7: Membrane bound O-acyltransferase domain containing 7; SAMM50: Sorting and assembly machinery component 50 homolog; COL13A1: Collagen, type XIII, alpha 1.

risk score constructed by summing the number of risk alleles for 6 different SNPs, among which PNPLA3 rs738409, was strongly associated with ALT levels in a study on 178 Mexican NAFLD patients<sup>[39]</sup>. Combining genetic information has proved effective also in predicting the risk of NAFLD<sup>[40,41]</sup>. In a study on 384 ultrasonographically-diagnosed NAFLD patients and an equal number of controls, an easy score based on the number of risk alleles for the PNPLA3 rs738409 and TM6SF2 rs58542926 SNPs, was significantly associated with the risk of NAFLD, with an average 1.52 increase in OR for each additional risk allele<sup>[40]</sup>. In another work, a 4-SNPs weighted genetic risk score based on the PNPLA3 rs738409, TM6SF2 rs58542926, GCKR rs1260326 and MBOAT7 rs641738 SNPs, was strongly associated again with ultrasonographically-diagnosed NAFLD independently from age, gender, body mass index (BMI), and homeostasis model assessment for insulin resistance (HOMAIR)<sup>[41]</sup>.

Much more interestingly, information gained from genetics has been shown to predict also the risk or presence of NASH, advanced fibrosis and even HCC<sup>[42-45]</sup>. Nobili *et al*<sup>[42]</sup> evaluated the diagnostic accuracy of a multivariate model based on 4 SNPs, *i.e.*, PNPLA3 rs738409, Krueppel-like factor 6 (KLF6) rs3750861, superoxide dismutase 2 (SOD2) rs4880 and Lipin1 (LPIN1) rs13412852, to predict the presence of NASH in 152 children with biopsy-proven NAFLD. The model significantly predicted NASH [area under the curve (AUC) = 0.75], performing better than a clinical risk score based on age, AST levels and diastolic blood pressure<sup>[42]</sup>. Interesting results have been obtained also in the adult population. When a genetic risk score based on 4 SNPs, among which PNPLA3 rs738409 and GCKR rs1260326, was evaluated in a population of 130 morbidly obese Mexican individuals undergoing liver biopsy at the time of bariatric surgery, it was not able to predict NASH (AUC = 0.56,  $P = 0.2$ ), but above a

**Table 2** Overview of the main studies testing combined genetic/clinical risk score in the assessment of non-alcoholic fatty liver disease patients

Ref.	Variables included	Risk score formula	Outcomes
Kotronen <i>et al</i> <sup>[46]</sup> , 2009	Clinical and laboratory data (metabolic syndrome, type 2 diabetes, insulin, AST, AST/ALT ratio and PNPLA3 rs738409 GG)	NAFLD liver fat score: $[-2.89 + 1.18 \times \text{metabolic syndrome (yes = 1/no = 0)} + 0.45 \times \text{type 2 diabetes (yes = 2/no = 0)} + 0.15 \times \text{fS-insulin (mU/L)} + 0.04 \times \text{fS-AST (U/L)} - 0.94 \times \text{AST/ALT}]$ and PNPLA3 rs738409 GG	Independent predictor of NAFLD with AUROC of $0.872 \pm 0.02$ (95%CI: 0.84-0.91); Addition of rs738409 to the score improved the accuracy of the prediction by only < 1%.
Franque <i>et al</i> <sup>[47]</sup> , 2012	ALT, fasting levels of C-peptide, ultrasound steatosis scores and PNPLA3 rs738409 genotypes	ND	Predictor of NASH with AUROC of 0.8 Rs738409 correlated with development of NASH but did not add value
Guichelaar <i>et al</i> <sup>[55]</sup> , 2013	PNPLA3 rs738409 G allele, CK-18 > 145 IU/L, Glucose > 100 mg/dL, C-reactive protein > 0.8 mg/dL	Sum of risk factors	82% probability of NASH (all four risk factors) <i>vs</i> 9% in their absence
Hyssalo <i>et al</i> <sup>[56]</sup> , 2014	PNPLA3 genotype, AST, fasting insulin	NASH score: $-3.05 + 0.562 \times \text{PNPLA3 genotype (CC = 1/GC = 2/GG = 3)} - 0.0092 \times \text{fS-insulin (mU/L)} + 0.0023 \times \text{AST (IU/L)} + 0.0019 \times (\text{fS-insulin} \times \text{AST})$	Finnish cohort NPV 86%, PPV 53% for NASH Italian cohort NPV 74%, PPV 65% for NASH
Zhou <i>et al</i> <sup>[48]</sup> , 2016	Glutamate, isoleucine, glycine, lysophosphatidylcholine 16:0, phosphoethanolamine 40:6, AST, and fasting insulin and	NASH ClinLipMet score: $-8.167 + 0.954 \times \text{PNPLA3 genotype (CC = 1/GC = 2/GG = 3)} + 0.0451 \times \text{AST (IU/L)} + 0.0667 \times \text{fS12 insulin (mU/L)} - 3.151 \times \log_{10}(\text{LysoPC(16:0)}) (\mu\text{mol/L}) + 2.617 \times \log_{10}(\text{PE(40:6)}) (\mu\text{mol/L}) + 2.357 \times 13 \log_{10}(\text{Glu}) (\mu\text{mol/L}) + 7.813 \times \log_{10}(\text{Ile}) (\mu\text{mol/L}) - 6.102 \times \log_{10}(\text{Gly}) (\mu\text{mol/L})$	Identified patients with NASH with an AUROC of 0.866 (95%CI: 0.820-0.913)
Donati <i>et al</i> <sup>[28]</sup> , 2017	PNPLA3rs738409 genotypes PNPLA3 rs738409, TM6SF2 rs58542926 and MBOAT7 rs641738, age, sex, obesity, type 2 diabetes, severe fibrosis	HCC risk score: $1/[1 + e^{-\{(-12.588 + (0.162 \times \text{age}) + (0.404 \times \text{sex: 1 if male, -1 if female}) + (0.259 \times \text{obesity: 1 present, -1absent}) + (0.587 \times \text{T2DM: 1 present,-1 absent}) + (1.299 \times \text{severe fibrosis: 1 yes, -1 no}) + (0.442 \times \text{number of risk alleles})\}}]$	Identified patients with HCC with an AUROC of $0.96 \pm 0.04$ (96% sensitivity, 89% specificity)

PNPLA3: Patatin-like phospholipase domain-containing protein 3; AUROC: Area under the receiver operating characteristic curve; CI: Confidence interval; NASH: Non-alcoholic steatohepatitis; TM6SF2: Transmembrane 6 superfamily member 2; ALT: Alanine aminotransferase; GGT:  $\gamma$ -glutamyl transferase; NAFLD: Non-alcoholic fatty liver disease; AST: Aspartate aminotransferase; MBOAT7: Membrane bound O-acyltransferase domain containing 7; HCC: Hepatocellular carcinoma; NPV: Negative predictive value; PPV: Positive predictive value.

specific threshold it was associated with a significant increase in the risk of NASH itself [odd ratio (OR) = 2.55,  $P < 0.05$ ]<sup>[43]</sup>. In a population of 416 biopsy-proven NAFLD patients, Koo *et al*<sup>[44]</sup> constructed a genetic risk score by counting risk alleles for PNPLA3 rs738409 (by coding 0, 1 and 2 for CC, CG and GG genotypes, respectively) and TM6SF2 rs58542926 (by coding 0 and 1 for CC and CT/TT genotypes, respectively). The genetic risk score was significantly associated with the risk of NASH (OR approximately 2 per risk allele) and fibrosis  $\geq$  F2 (OR approximately 2 per risk allele)<sup>[44]</sup>. Recently, we evaluated a series of NAFLD patients with a large component of NASH-cirrhotics and, consistent with the associations in baseline statistics, we selected 3 polymorphisms to build a genetic risk score (PNPLA3 rs738409, TM6SF2 rs58542926 and Kruppel-like factor6 -KLF6- rs3750861)<sup>[45]</sup>. Notably, when compared to a score of 0, a genetic risk score  $\geq$  3 almost quadrupled the risk of NASH cirrhosis in NAFLD patients<sup>[45]</sup>. Lastly, Donati *et al*<sup>[28]</sup> evaluated a population of 132 patients with NAFLD-HCC and reported a significant association between the number of risk alleles, among PNPLA3 rs738409, TM6SF2 rs58542926 and MBOAT7 rs641738 SNPs, and the risk of HCC (OR per allele approximately 1.6).

Although all these results appear encouraging, the overall experience suggests that the value of utilizing genetic variants for improving diagnostic accuracy in the context of NAFLD is still inconclusive. Kotronen *et al*<sup>[46]</sup> evaluated the accuracy of a NAFLD liver fat score, built by multivariate logistic regression analyses and including the diagnosis of metabolic syndrome and T2DM plus insulin, AST and ALT levels, to predict liver fat content as evaluated by proton magnetic resonance spectroscopy. The score displayed a sensitivity of 86% and a specificity of 71% in the prediction of increased liver fat content; however, when the genetic information constituted by the PNPLA3 rs738409 genotype was included in the model, the prediction accuracy improved by only 1%<sup>[46]</sup>. A similar result was obtained by Franque *et al*<sup>[47]</sup>, who reported that the PNPLA3 variant was significantly associated with NASH but did not increase the diagnostic accuracy of a model based on routine clinical parameters. More positive are the results reported by Zhou *et al*<sup>[48]</sup>, who created the NASH ClinLipMet Score by combining plasma metabolites (glutamate, isoleucine, glycine, etc), routine biochemical test (AST, fasting insulin) and the PNPLA3 rs738409 genotype, and demonstrated its good diagnostic accuracy for the diagnosis of NASH (AUC approximately 0.87).

## THE FUTURE OF GENETICS IN THE ASSESSMENT OF NAFLD PATIENTS: HOW TO FAVOR CLINICAL TRANSLATION?

As discussed, in the last years, a number of genetic variants have been associated with NAFLD and, more recently, there have been also attempts to combine them in pure genetic or combined genetic/clinical scores. However, to date, clinical translation has been poor or absent.

First of all, we need to make order. Almost all the studies produced to date presented a cross-sectional design and were aimed to evaluate either the increase of the risk or the implementation of diagnostic accuracy for the different NAFLD-related outcomes determined by the presence of a specific polymorphism or a combination of more of them. In the first case, demonstrating that a polymorphism is associated with a statistically significant increase of the risk of NAFLD, NASH, NASH-fibrosis or NAFLD-HCC does not mean giving concrete predictive information of clinical utility for the single patient. In the second case, proving that the addition of one or more variants to a model increase its diagnostic accuracy for an outcome which has already occurred means using the genetic information equally to any other clinical or biochemical one. Although this could be of aid, it is not exactly the best we can gain from genetics. Indeed, unlike classical risk prediction, genetic risk prediction is highly stable over time and can be performed on a much longer time scale. Therefore, the risk can be stratified even before the disease occurs or progress, allowing monitoring and interventions to be started at an early age. However, for this aim, data should be clearly obtained from longitudinal studies.

Secondly, we need to focus on relevant outcomes. It seems difficult that a variant which is associated only with the risk of NAFLD, and not with that of its fibrotic evolution, deserves clinical translation. Indeed, genetic or combined risk scores are not expected to be applied at a general population level to predict the risk of NAFLD; rather, they are clearly awaited with the aim to help stratifying the risk of disease progression in patients already diagnosed with NAFLD. In this context, we are favored by the fact that most of the variants which have been initially associated with liver fat content, *i.e.*, PNPLA3 rs738409, TM6SF2 rs58542926, and MBOAT7 rs641738, have been concurrently or subsequently associated also with NASH and fibrosis<sup>[16,17,23,28,32]</sup>. This is consistent with the almost 80% shared heritability which has been described between hepatic fat content and fibrosis<sup>[15]</sup>, and confirms that fat accumulation is an important driver of disease progression.

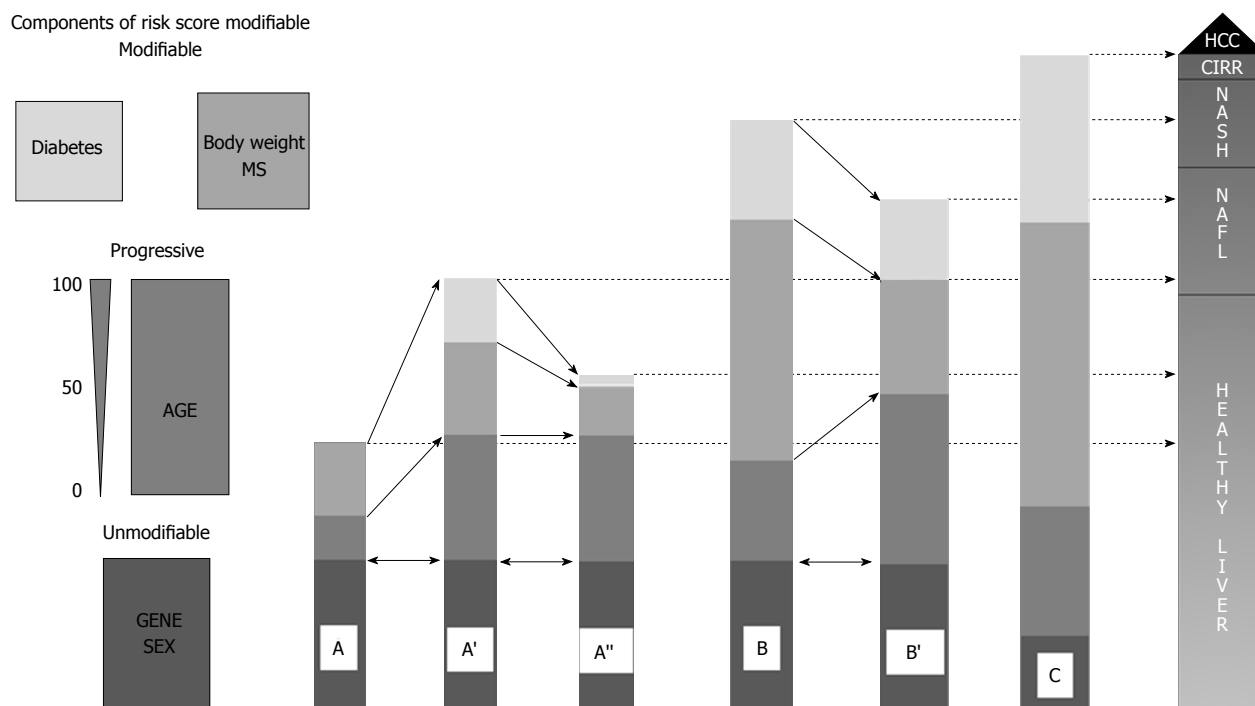
The evidence produced has also taught us what we should expect from genetics in terms of prediction. Indeed, while approximately half of hepatic fat content variability is explained by genetic factors<sup>[15-18]</sup>, the

other half has acquired determinants. Moreover, in these 50% of genetic predisposition included are both: the few well-known polymorphisms responsible for a strong variance, (*i.e.*, approximately 5% in the case of PNPLA3 rs738409<sup>[18]</sup>), as well as a number of other polymorphisms, individually responsible for a much lower variance, which remain unknown due to the fact that they do not reach a genome-wide significance. Finally, very rare variants with a strong phenotypic impact may be the principal driver in specific cases. Hopefully, focusing on relevant outcomes, *i.e.*, advanced fibrosis, cirrhosis decompensation or liver-related death, we will probably observe an increase of the variance attributable at least to some of the polymorphisms already known. For example, if approximately 5% of the variance in hepatic fat content has been attributed to PNPLA3 rs738409<sup>[18]</sup>, it seems probable that a much higher percentage of variance will be attributed to this same polymorphism with respect to the evolution to NASH-cirrhosis and NAFLD-related HCC. Indeed, while the prevalence of homozygosity for the mutated PNPLA3 allele is about 5% in the general population and 15%-20% in NAFLD patients, it goes up to 30%-50%, according to the different series, in patients with NASH-cirrhosis or NAFLD-related HCC<sup>[19,28,49]</sup>, suggesting that the variant is even more important for the physiopathologic mechanisms involved in disease evolution than in disease development.

Another central issue to consider when approaching this field is the large gene-environment interaction. Indeed, soon after the discovery of the PNPLA3 variant, it was demonstrated that its effect was strongly amplified in subjects with obesity<sup>[50]</sup>. More recently, Stender *et al.*<sup>[51]</sup> comprehensively evaluated this aspect again for PNPLA3 and for other two polymorphisms, *i.e.*, TM6SF2 rs58542926 and GCKR rs1260326, and demonstrated that adiposity significantly potentiates the effect of these three variants, suggesting that the synergy between adiposity and genetics can promote the full spectrum of NAFLD, from steatosis to NASH and cirrhosis. Notably, in adolescents, a significant interaction has been described between the PNPLA3 variant and dietary components, in particular intake of sweetened beverages, with respect to steatosis severity<sup>[52]</sup>. Considering this strong gene-environment interaction, pure genetic scores are not likely to reach a sufficient predictive power, while combined genetic/clinical ones, including variables which have been consistently associated with disease severity, *i.e.*, age, BMI and T2DM, will more probably reach this goal.

Altogether, we hope that combined genetic/clinical scores, derived from longitudinal studies and built on a few strong genetic variants and relevant clinical variables, will reach a significant predictive power, such as to have clinical utility for risk stratification at the single patient level. This kind of scores may have also the advantage to be dynamic, modifying with the variation of clinical variables over time, and permitting to esteem the impact





**Figure 2 Combined genetic/clinical risk score built through a few strong gene variants and relevant clinical variables.** Genetic background and sex (unmodifiable), age (progressive), the components of metabolic syndrome (with diabetes in a pre-eminent position-modifiable-) could be weighed and combined to make up an individual risk score. This type of score can have the advantage of being dynamic, changing as the clinical conditions change over time, and allowing to estimate the impact of intervention strategies on the onset/outcomes of the disease. In the first column, patient A has an unfavorable genetic background; he is 20 years old, he is normal weighted, and he is not diabetic. The risk score changes when he is in his 60s, is lightly overweight and with reduced glucose tolerance (column A'). In column A'', the same patient in his 60s but has made efforts to stay lean and with a better glycemic metabolism (intervention). Column B and B' represent the score of a patient who has an unfavorable genetic background; he is in his 40s, he is obese and he has impaired glucose metabolism. The risk score changes when he is in his 70s but made efforts to stay lean and with a slightly better glucose tolerance (intervention strategy). Column C represents the individual score of a patient with a favorable genetic background combined with with overweight and diabetes as protagonists. CIRR: Cirrhosis; GENE: Genetic background; HCC: Hepatocellular carcinoma; MS: Metabolic syndrome; NAFL: Non-alcoholic fatty liver; NASH: Non-alcoholic steatohepatitis.

**Table 3 Primary and secondary objectives for which combined genetic/clinical scores are expected in the field of non-alcoholic fatty liver disease**

Primary objectives	Prediction of hard outcomes (cirrhosis decompensation, hepatocellular carcinoma, liver transplantation)
	Prediction of fibrosis progression
Secondary objectives	Complementary instruments for the diagnosis of NASH and/or advanced fibrosis
	Prediction of response to lifestyle interventions
	Prediction of response to pharmacologic interventions

NASH: Non-alcoholic steatohepatitis.

of intervention on the risk of disease-related outcomes (Figure 2). Adequate patient populations from which to draw with the aim to construct these models could be those in the placebo-arm of clinical trials with the new drugs tested for their efficacy in NASH. Indeed, in these studies, patients are assessed with histology at baseline, when DNA for the genetic information is frequently collected, and re-evaluated over time, in some cases with long follow-up focused on hard outcomes further than on fibrosis evolution over time. The scores created by this way could therefore be tested against baseline histology

for their accuracy to diagnose NASH and/or significant fibrosis and, mainly, be evaluated with respect to their capability to predict fibrosis progression and adverse outcomes. Finally, verifying the accuracy of the scores to predict the impact of specific interventions in the active arm of the same trials seems particularly intriguing (Table 3). Well-structured future studies would demonstrate if this vision can become a reality.

## CONCLUSION

In the last years, many genetic variants have demonstrated a central role in NAFLD development and evolution, and have even helped us to understand important steps in NAFLD pathophysiology. This evidence suggests that genetics should be included in combined predictive models which, together with relevant clinical variables, would help to stratify the risk at the single patient level and, finally, to orient medical care and resources in the NAFLD epidemic. All that we have done has not brought us to the goal yet, but it seems to have placed us on the right path.

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