Review

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Update on NAFLD genetics: From new variants to the clinic

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the leading cause of liver diseases in high-income countries and the burden of NAFLD is increasing at an alarming rate. The risk of developing NAFLD and related complications is highly variable among individuals and is determined by environmental and genetic factors. Genome-wide association studies have uncovered robust and reproducible associations between variations in genes such as *PNPLA3*, *TM6SF2*, *MBOAT7*, *GCKR*, *HSD17B13* and the natural history of NAFLD. These findings have provided compelling new insights into the biology of NAFLD and highlighted potentially attractive pharmaceutical targets. More recently the development of polygenic risk scores, which have shown promising results for the clinical risk prediction of other complex traits (such as cardiovascular disease and breast cancer), have provided new impetus for the clinical validation of genetic variants in NAFLD risk stratification. Herein, we review current knowledge on the genetic architecture of NAFLD, including gene-environment interactions, and discuss the implications for disease pathobiology, drug discovery and risk prediction. We particularly focus on the potential clinical translation of recent genetic advances, discussing methodological hurdles that must be overcome before these discoveries can be implemented in everyday practice.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the leading cause of liver disease in high-income countries, affecting more than 25% of the population.[1] The inflammatory form of this condition, namely non-alcoholic steatohepatitis (NASH), is responsible for an increasing proportion of cases of cirrhosis and hepatocellular carcinoma (HCC).[2,3] As for other complex traits (*e.g.* circulating lipids or cardiovascular diseases), the risk of developing NAFLD and NASH varies among individuals; it is ultimately determined by the combination of environmental factors, such as adiposity or the presence of type 2 diabetes (T2D), and inherited genetic variations.

Historically, genetic susceptibility to NAFLD has been evaluated using candidate gene studies – a study design evaluating the association of variants in a given gene and a phenotype of interest. However, most candidate gene studies have only identified and validated a handful of loci associated with the risk of NAFLD prevalence or progression. This can be explained by the limited number of genes selected a priori based on their plausible biological relevance, but also by various methodological drawbacks (*e.g.* limited statistical power).[4] Conversely, genome-wide association studies (GWAS) test the association of millions of variants throughout the genome in an unbiased fashion. GWAS can be performed using various technologies, like single nucleotide polymorphism (SNP) arrays followed by imputation, a statistical method for inferring genotypes that are not directly measured using large reference panels (*e.g.* 1000G or the Haplotype Reference Consortium).[5,6] More recently, next-generation sequencing approaches have been implemented, including whole-exome sequencing (WES), targeting the fraction of the genome that encodes proteins, or whole-genome sequencing (WGS). GWAS have identified robust and reproducible associations linked with the natural history of NAFLD, including variants in the patatin-like phospholipase domain-containing 3 (*PNPLA3*), the transmembrane 6 superfamily member 2 (*TM6SF2*) and more recently in the 17-beta hydroxysteroid dehydrogenase 13 (*HSD17B13*) genes.[7] GWAS have uncovered novel NAFLD susceptibility genes and biological pathways and fostered improved understanding of NAFLD pathophysiology.

Herein, we provide an update on our current knowledge of NAFLD genetics and discuss the benefits and limitations of recent GWAS findings including biological understanding, risk prediction and drug development. Based on the available evidence, we propose suggestions for interpretation, and design of new studies in the field. We will frequently use the more general term fatty liver disease (FLD), as at risk alcohol intake was not an exclusion criterium in most studies, and it is always

difficult to differentiate the role of alcohol and metabolic risk factors.[8] Furthermore, genetic studies have highlighted shared inherited determinants of metabolic and alcohol-related FLD.[9]

Current knowledge on the genetics of NAFLD

Heritability of NAFLD

Heritability is defined as the proportion of phenotypic variation in a trait that is due to genetic variation.[10] Unlike monogenic diseases, such as hereditary haemochromatosis and Wilson's disease, the heritability of complex traits involves thousands of common genetic variants (minor-allele frequency [MAF] ≥5%) distributed throughout the genome, which are usually characterised by small effect sizes (*e.g.* relative risks or odds ratios [ORs]).[11]

A large fraction of hepatic fat and FLD variability in the population, ranging from 25% to 75%, is accounted for by inherited factors.[9] This evidence is supported by studies in twins, showing 50% heritability of FLD, as estimated by aminotransferases and more recently by direct evaluation of hepatic fat content.[12,13] The implementation of nuclear magnetic resonance approaches to measure liver fat and fibrosis by elastometry revealed that these traits are co-inherited in the population.[13] These results are in line with the hypothesis that quantitative/qualitative alterations of hepatic fat cause progressive liver disease.[14,15] Multi-ethnic cohorts have also highlighted a major inter-ethnic variability in FLD susceptibility: higher in Hispanics, intermediate in Europeans and lower in individuals of African descent, independently of confounders.[16] In family studies, the risk of severe liver fibrosis was 12.5-fold higher in first-degree relatives of patients with NAFLD-related cirrhosis (18%) compared to the general population (1%), independently of dysmetabolism.[17] A family history of NAFLD is associated with a higher risk of this condition, in particular when both parents are affected.[18] Therefore, the first practical message is that ethnicity and family history should be recorded, because they have a clinically relevant impact on both FLD development and progression.

Genetic loci associated with NAFLD

Findings from GWAS conducted in large cohorts of well-phenotyped individuals enabled the identification of the first and main FLD risk variants that are common in the population.[19–22] The list of common variants associated with NAFLD and NASH, which were independently validated in large multicentre cohort, and whose impact on liver disease was supported by functional studies are presented in Table 1.

The rs738409 C>G SNP, encoding the I148M variant of *PNPLA3*, accounts for the largest fraction of genetic predisposition to NAFLD.[19] Carriage of the I148M variant facilitates hepatic fat accumulation, without a major direct impact on adiposity and insulin resistance.[19] Major findings were that the *PNPLA3* I148M variant increases susceptibility to the whole spectrum of liver damage related to NAFLD, from steatosis, to NASH, fibrosis, and HCC,[23–27] and is a common modifier of liver disease risk.[28–32] Carriage of the I148M variant has been associated with an increased risk of liver-related mortality in patients with NAFLD and in the general population.[33,34]

Key point

Growing evidence has shown that the risk of NAFLD occurrence and progression varies among individuals and has highlighted the role of inherited genetic variants.

The rs58542926 C>T that codes for the E167K variant of *TM6SF2* favours hepatic fat accumulation in intracellular lipid droplets by decreasing lipid secretion, thereby leading to increased susceptibility to liver damage, including NASH and severe fibrosis. At the same time, the E167K variant protects against cardiovascular disease by reducing circulating lipids.[21,35,36] However, it predisposes individuals to HCC development.[37] The rs641738 C>T variant close to the membrane bound O-acyltransferase domain-containing 7 *(MBOAT7)* locus was identified as a risk factor for alcohol-related cirrhosis,[28] and is associated with the predisposition to accumulate fat in the liver and to develop NAFLD, inflammation, fibrosis, and HCC, due to reduced protein expression.[27,38]

Variation at the glucokinase regulator (*GCKR*) gene locus has also been associated with NAFLD.[14,20,39] A common missense variant (rs1260326), encoding P446L, is most likely the causal variant underlying the association.[40] The protein phosphatase 1 regulatory subunit 3B (*PPP1R3B*) rs4841132 variant has also been suggested to protect against hepatic fat accumulation[20,41,42] by modulating lipid synthesis.[41] However, the overall impact on the risk of liver-related events remains controversial.[42]

Activation of innate immunity and fibrogenesis modulate disease progression in patients with NAFLD. The rs368234815 δG>TT and linked variants encoding for interferon-λ4 (*IFNL4*) instead of the IFNL3 protein, have been associated with decreased expression of interferon-stimulated genes, but more severe inflammation and fibrosis.[43,44] Variation in Mer T kinase (*MERTK*) affect inflammation and fibrosis, as the protein, a membrane tyrosine kinase receptor, regulates the activation of phagocytes and hepatic stellate cells.[43] The *MERTK* rs4374383 variant protects against fibrosis development by reducing hepatic MERTK expression.[43,45] Another variant possibly associated with liver damage and inflammation is rs236918 in proprotein convertase subtilisin/kexin type 7 (*PCSK7*), which modulates multiple pathways, including lipid and iron metabolism as well as fibrogenesis.[46]

The *HFE* C282Y variant of hereditary hemochromatosis, encoded by the rs1800562 polymorphism, is a major determinant of liver damage and cirrhosis risk in Europeans.[47,48] Its impact on liver damage in patients with NAFLD/NASH is still

Key point

GWAS in the field of NAFLD have identified robust and reproducible associations for variants in *PNPLA3*, *TM6SF2*, *MBOAT7*, *GCKR* and *HSD17B13*, all contributing to a better understanding of NAFLD biology.

Table 1. List of common variants associated with NAFLD/NASH.

Gene	Variant	Impact on protein	Effect of the variant	Allelic frequency Europeans	Hispanics	Asians	Africans	Effect size	Effect of the variant	Direction of association (Ancestral allele)	Fat	NASH	Fibrosis	HCC	Mortality	Response to therapies
PNPLA3	rs738409 C>G	I148M	Complex: loss- plus gain-of-function	0.23	0.57	0.38	0.14	+++	Complex: loss plus gain of function	↑	+	+	+	+	+	+
TM6SF2	rs58542926 C>T	E167K	Loss-of-function	0.08	0.03	0.07	0.04	+++	Loss-of-function	↑	+	+	+	+		+
GCKR	rs1260326 T>C	P446L	Loss-of-function	0.60	0.67	0.50	0.86	+	Loss-of-function	↑	+	+	+			
MBOAT7	rs641738 C>T	Linked to 3'-UTR	Reduced expression	0.42	0.33	0.24	0.34	+	Reduced expression	↑	+	+	+	+		
HSD17B13	rs72613567 T>TA rs62305723 G>A	Alternate splicing P260S	Loss-of-function	0.27 0.07	0.09 0.02	0.34	0.06 0.01	++	Loss-of-function	↓		+	+	+		
IL28B (IFNL3/4)	rs368234815 TT>δG	Alternate protein translation site	Alternative protein	0.27	0.09	0.34	0.06	+	Alternative protein	↓		+	+			
MERTK	rs4374383 G>A	Noncoding variant	Reduced expression	0.37	0.37	0.73	0.47	+	Reduced expression	↓			+			

We considered variants identified through unbiased genome-wide/exome-wide association studies, which were independently associated with the development and/or progression of NAFLD/NASH in multicentre or independent studies, and for which there is evidence of a mechanism linking the variant with disease predisposition. NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

debated and probably depends on its impact on iron accumulation.[49,50] This variant causes liver damage by promoting oxidative stress, which can also be modulated by variants in the nuclear genome-encoded mitochondrial proteins *SOD2*, *UCP2*, and *MARC1*,[47,51,52] whose impact on liver disease risk needs further confirmation.

Recently, loss-of-function variants in *HSD17B13*, which encodes an enzyme that localises to lipid droplets in hepatocytes, have been linked to robust protection against liver inflammation, cirrhosis, and HCC due to both dysmetabolism and alcohol.[53,54] The mechanism linking *HSD17B13* variants with liver disease is not related to hepatic fat accumulation, but involves direct modulation of inflammation and fibrogenesis.[22,55,56]

Breakthroughs in NAFLD pathobiology

GWAS have highlighted the role of lipid droplet biology, intracellular lipid synthesis and degradation, and secretion of very low-density lipoproteins (VLDLs) in the pathogenesis of NAFLD, and have identified new players involved in these processes (Fig. 1). These discoveries have provided a solid foundation from which to improve our understanding of the pathogenesis of liver disease, and have been validated by functional studies and by wide replication in clinical cohorts.[57]

The discovery of PNPLA3 has transformed our understanding of fatty liver, shifting the attention to lipid remodelling in intracellular droplets as the common pathway underlying disease progression irrespective of the environmental trigger. *PNPLA3* is induced by insulin in hepatocytes, hepatic stellate cells, and adipocytes during insulin resistance.[58,59] The wild-type PNPLA3 is involved in the remodelling of triglycerides, phospholipids, and in the release of retinyl-esters, by acting as a lipase on lipid droplets.[59,60] While the wild-type protein is rapidly degraded, the variant protein has no lipase activity and accumulates, impairing lipid remodelling and turnover.[60–63] These alterations require the sequestration of ABHD5/CGI-58, an essential cofactor for ATGL/PNPLA2, the major lipid droplet lipase in hepatocytes,[64,65] and may involve the impairment of lipo-autophagy.[66] Enlargement and altered qualitative composition of lipid droplets then trigger lipotoxicity. This gain-of-function model involving the trans-repression of ATGL as an explanation for the impact of the I148M variant[60,61,64,67] is supported by human genetics, in that variation at the *PNPLA3* locus associated with lower *PNPLA3* expression curbed the phenotype of the I148M variant,[60–62] while loss-of-function *PNPLA3* variants were not associated with severe liver disease.[37] However, the I148M variant also leads to altered lipid remodelling, with accumulation of polyunsaturated fatty acids in diacylglycerol and triglycerides, and a parallel depletion in phospholipids, which depends on altered enzymatic activity.[61,63] The detrimental impact on

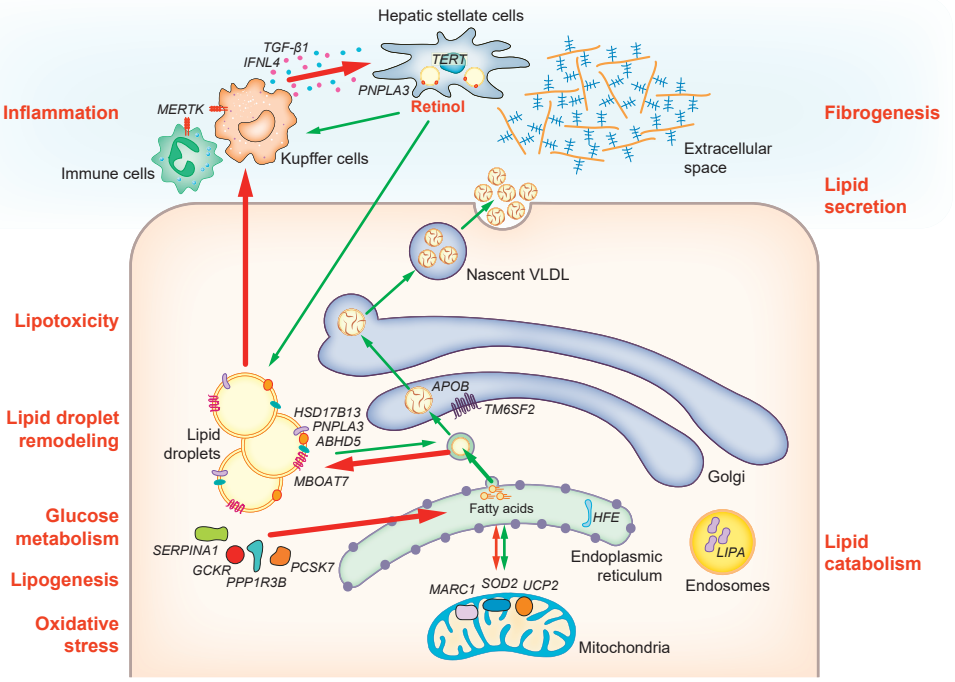

Fig. 1. Genetic pathophysiology of NAFLD. Genetic determinants of FLD, classified according to the biological processes by which the encoded proteins are thought to contribute in the pathogenesis of the disease in the liver. Red arrows indicate pathological processes/lipid fluxes, while green arrows beneficial pathways. Pathophysiological processes are indicated in red uppercase, gene names in Italics, cellular and liver compartments in lowercase. APOB, apolipoprotein B; FLD, fatty liver disease; GCKR, Glucokinase regulator; HFE, haemochromatosis gene; HSD17B13, 17-beta hydroxysteroid dehydrogenase 13; IFNL4, interferon lambda 4; LIPA, lysosomal acid lipase; NAFLD, non-alcoholic fatty liver disease; MARC1, mitochondrial amidoxime reducing component 1; MBOAT7, membrane bound O-acyl transferase 7; MERTK, Mer T kinase; PCSK7, proprotein convertase subtilisin/kexin 7; PNPLA3, patatin-like phospholipase domain-containing 3; PPP1R3B, protein phosphatase 1 regulatory subunit 3B; SOD2, mitochondrial superoxide dismutase; TERT, human telomerase reverse transcriptase; TM6SF2, transmembrane 6 superfamily member 2; UCP2, uncoupling protein 2; VLDL, very low-density lipoproteins.

adipocyte function and the secretion of adiponectin may contribute to the liver phenotype associated with the I148M variant.[65,68]

A key role of impaired lipid droplet degradation in NAFLD is supported by the phenotype or rare variants identified through classic family-based genetic studies and the application of next-generation sequencing to the diagnosis of unexplained NAFLD or cryptogenic liver disease. Indeed, heterozygous carriage of mutations that cause impaired protein activity of abhydrolase-containing domain 5 (*ABHD5*) – a direct binding partner of PNPLA3 and ATGL – result in severe NAFLD.[69] Furthermore, lysosomal acid lipase deficiency, caused by mutations of the *LIPA* gene, causes a severe genetic form of NAFLD. The mechanism is related to the accumulation of cholesteryl esters and triglycerides in hepatocytes due to defective lysosomal hydrolysis and lipo-autophagy.[70]

The importance of phospholipid remodelling in NAFLD is independently supported by the role of *MBOAT7* in disease predisposition. Indeed, MBOAT7 is involved in the remodelling of phosphatidylinositol and other phospholipids by incorporating arachidonic acid and other unsaturated fatty acids into lysophospholipids. The common rs641738 C>T variant that predisposes to liver disease leads to *MBOAT7* downregulation,[27] and reduced levels of arachidonic acid bound to phophatidyl-inositol.[38,71] Saturated lyso-phosphatidyl-inositol accumulates and is diverted to the synthesis of triglycerides. Downregulation of hepatic MBOAT7 is implicated in NAFLD development during obesity and insulin resistance.[72,73] A role for qualitative alterations in lipid droplet remodelling is also supported by the fact that HSD17B13 is predicted to metabolise several lipid species.[55]

Regulation of the flux of lipids from intracellular droplets to the synthesis and secretion of VLDL are also involved in hepatic fat accumulation and consequent liver disease. This concept is highlighted by the mechanism underlying NAFLD development in carriers of the *TM6SF2* E167K variant. In humans, TM6SF2 regulates qualitative triglyceride enrichment in VLDL, but also lipid synthesis and the number of secreted lipoprotein particles, while E167K is a loss-of-function variant favouring lipid compartmentalisation into the liver.[74,75] Mendelian disorders again support this interpretation. Homozygous familial hypobetalipoproteinemia, caused by rare mutations in apolipoprotein B (*APOB*), predisposes individuals to severe progressive liver disease due to the inability of hepatocytes to secrete VLDL.[76] Furthermore, carriage of *APOB* mutations in heterozygosity has recently been associated with increased risk of developing HCC related to NAFLD.[37] Unlike abetalipoproteinemia, which is caused by bi-allelic loss-of-function mutations in microsomal triglyceride

transfer protein (*MTTP*) and is also associated with liver damage, this condition was less frequently associated with severe malabsorption and more frequently with development of obesity in adulthood.[37] It has also recently emerged that part of the impact of carriage of the *SERPINA1* PiZ (but not PiS) variant responsible for alpha-1 antitrypsin deficiency (rs28929474) on liver damage may be related to altered lipid secretion and fatty liver due to endoplasmic reticulum stress.[77] Modulation of the rate of lipid synthesis may also have a role. Indeed, the P446L *GCRK* variant acts by hampering the negative feedback inhibition of fructose-6-phosphate on glucokinase, thereby removing the brakes on malonyl-CoA synthesis and consequently *de novo* lipogenesis in response to circulating glucose.[40]

Key point

The number of loci currently associated with NAFLD is still limited and the identification of new variants will require larger collaborative efforts to perform powerful GWAS.

Recent findings also point to a possible role of the impairment of retinol release from lipid droplets of hepatic stellate cells, with subsequent conversion to retinoic acid acting on inflammation, fibrogenesis, and carcinogenesis, in mediating FLD predisposition in carriers of the *PNPLA3* I148M variant.[78–80] This process may be dependent on the direct induction of a pro-inflammatory and pro-fibrogenic phenotype in hepatic stellate cells.[80,81] Furthermore, *HSD17B13* variants which protect against NAFLD determine a reduced activity or mislocalisation of the enzyme, which is involved in the conversion of retinol to retinoic acid in lipid droplets in hepatocytes.[55] Finally, retinoic acid supresses fibrogenesis in NAFLD due to its ability to induce the cleavage and inactivation of MERTK in Kupffer cells, thereby reducing TGF-β1 release, activation of hepatic stellate cells and fibrogenesis.[45] As *MERTK* variation protects against fibrogenesis by reducing protein expression in Kupffer cells,[45] modulation of retinoic acid availability may represent another common genetic pathway of NAFLD.

Key point

Gene-environment and gene-gene interactions influence NAFLD prevalence and progression. The interaction between *PNPLA3* and body mass index seems particularly robust.

Therefore, quantitative and qualitative alterations to lipid content in hepatocytes drive NAFLD/NASH development and progression. As the metabolism of several lipid species and retinol are very different between humans and rodents, and mouse models have so far failed to fully recapitulate the phenotype of FLD risk variants, these findings highlight the necessity to find complementary approaches to study FLD pathophysiology. These may include 3D multilineage culture models of human cells and organoids.[82]

Considering the genetic architecture of NAFLD: practical implications

Genetic association findings in NAFLD are dependent on the study sample size

Recognition of the validity of GWAS for providing new insights into complex traits, as recently demonstrated for NAFLD, relies on the high reproducibility and robustness of their findings. However, this success comes at the cost of a stringent statistical significance threshold ($p < 5\times10^{-8}$) used in most GWAS to correct for the burden of multiple testing, as millions of SNPs throughout the genome are assessed.[83] The sample size required to detect a given genetic variant with suitable statistical power increases in inverse proportion to the effect size, and the frequency of the disease-causing allele.[84]

The available evidence suggests that the heritability of complex traits is mostly explained by numerous common variants (MAF ≥5%), the vast majority of them having small effect size.[11] Even after accounting for all known genetic factors, the inherited fraction of FLD susceptibility remains unexplained for >65%.[14] However, rare variants (MAF <1%) with large effect sizes are still expected to contribute,[85] as recently reported for NAFLD-related HCC,[37] but we must suppose that a large number of common genetic risk variants remain to be identified. The most straightforward strategy to overcome the current limitation in statistical power is to increase the sample size of study cohorts,[86] and this approach has proven successful in other complex liver diseases (Fig. 2). For each phenotype, the number of identified loci markedly increases above a certain threshold and this number has currently not reached a plateau in any complex disease.[87,88] The sample size in NAFLD GWAS published to date is relatively modest;[19–21] for instance, variants in *PNPLA3* or *TM6SF2* were captured because their effect size per risk allele is much larger than those usually reported in other complex traits (OR typically <1.3).[83] The growing availability of large publicly available databases linked to GWAS data, such as the UK Biobank,[88] will hopefully empower variant discovery.[47,48] Nevertheless, more large-scale collaborative efforts that systematically assess FLD in well-characterised cohorts are warranted, as increasing the sample size will inevitably result in the discovery of additional risk loci.

To date, the overwhelming proportion of GWAS have been published in individuals of European descent.[83] However, genetic architecture varies between populations of different ethnic background and current published GWAS findings may not be generalisable to other populations, as highlighted in a recent well-powered multi-ethnic GWAS of 26 clinical and behavioural phenotypes.[89] A strength of currently available FLD-GWAS has been the evaluation of multi-ethnic cohorts including individuals of African ancestry.[19,21] This led to the demonstration that *PNPLA3* I148M accounts for more than half of the inter-ethnic variability in the predisposition to develop FLD.[19] Of note, *PNPLA3* rs6006460[T] (S453I protein variant) was found to be associated with lower hepatic fat content and was common in African Americans (MAF = 0.104), but rare in European Americans (MAF = 0.003) and Hispanics (MAF = 0.008).[19] Similarly, a variant in *HSD17B13* (rs143404524, A192fs) which may confer loss-of-function, had a higher frequency in individuals of African ancestry

(MAF = 0.187) than in those of Hispanic (MAF = 0.024) or European (MAF = 0.002) descent.[90] The allelic frequency of the major common risk variants for NAFLD in different populations is reported in Table 1. These findings imply that future GWAS will have to include diverse populations to increase the likelihood of capturing new risk variants for FLD.

Another approach to discover new genetic determinants of FLD may be represented by the validation of the association of this condition with candidate variants that have been robustly demonstrated to influence FLD-related traits. For example, *MBOAT7* variation was identified as a determinant of alcohol-related cirrhosis, while *GCKR* variation was a modulator of circulating glucose and triglycerides.[28,91]

Gene-environment and gene-gene interactions

As in other complex traits, NAFLD results from the interplay between environmental determinants (*e.g.* adiposity, type 2 diabetes) and genetic variations. The impact of a given variant may be modulated by the magnitude of an environmental factor of the studied trait (*i.e.* gene-environment interactions) or by the number of alleles of another genetic variant (*i.e.* gene-gene interactions).[92] This phenomenon may also account for a fraction of the missing heritability of NAFLD. However, the lack of robustly replicated gene-environment or gene-gene interactions, to date, did not point to a predominant influence on the risk of most complex traits.[93]

Gene-environment interactions in NAFLD have been reported in mouse models, where genetically modified mice expressing *PNPLA3* I148M did not develop steatosis when fed with a low-fat chow diet but experienced an increase in hepatic fat compared to wild-type mice when on a high-sucrose diet.[94] In humans, the interaction between adiposity and *PNPLA3* I148M has been demonstrated in two large cohorts from the general population.[95] The impact of the I148M variant on steatosis accumulation, inflammation (using alanine aminotransferase levels as a surrogate) and the risk of cirrhosis was modulated by body mass index (BMI) (Fig. 3). Most of the effect was observed for individuals who were obese (BMI of 30–35 kg/m^2) or very obese (BMI >35 kg/m^2), indicating that the impact of *PNPLA3* variation on the natural history of NAFLD is strongly dependent on BMI levels.[95] The authors also observed an interaction between BMI and *TM6SF2* E167K and *GCKR* P446L on steatosis accumulation. However, no interactions were detected for other BMI-associated phenotypes (*e.g.* circulating triglycerides), suggesting a robust and specific gene-environment interaction between FLD risk variants and adiposity.[95] The mechanism underlying FLD development in obese individuals at high genetic risk may be related to the development of insulin resistance and hyper-insulinemia.[96]

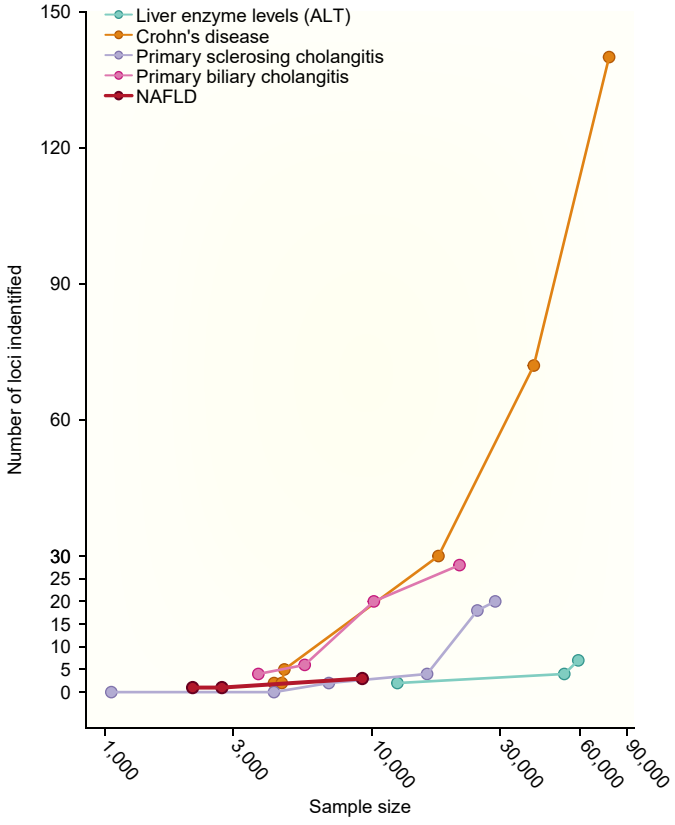

Fig. 2. Number of loci identified according to GWAS sample size. The number of independent loci reaching genome-wide significant loci (p <5×10^{-8}) from GWAS conducted in individuals predominantly of European descent in NAFLD,[19–21] liver enzyme levels (alanine aminotransferase),[22,39,128] primary biliary cholangitis,[129–132] primary sclerosing cholangitis[133–138] and Crohn's disease[139–144] increase according to the sample size (relevant summary statistics were downloaded from the NHGRI-EBI GWAS Catalog[83]). For each phenotype, the number of associated loci markedly increase above a certain threshold. ALT, alanine aminotransferase; GWAS, genome-wide association study; NAFLD, non-alcoholic fatty liver disease.

In addition, 30 SNPs previously linked to BMI were incorporated into a risk score that was also associated with hepatic fat, indicating that the genetic susceptibility to NAFLD goes beyond *PNPLA3*, *TM6SF2* and *GCKR* loci and also involves gene-gene interactions.[95] Overall, gene-environment and gene-gene (genetic epistasis) interactions modulate NAFLD promotion and progression and may therefore account for some of the unexplained heritability of NAFLD. Therefore, to increase the power to identify new genetic determinants of FLD, future studies should consider the interaction with environmental and genetic triggers or consider studying individuals at higher environmental or genetic risk. For example, one would expect that conducting GWAS in well-phenotyped insulin resistant patients not selected for liver damage – rather than individuals from the general population – would increase the statistical power to detect new variants, especially protective ones, that modulate FLD risk.

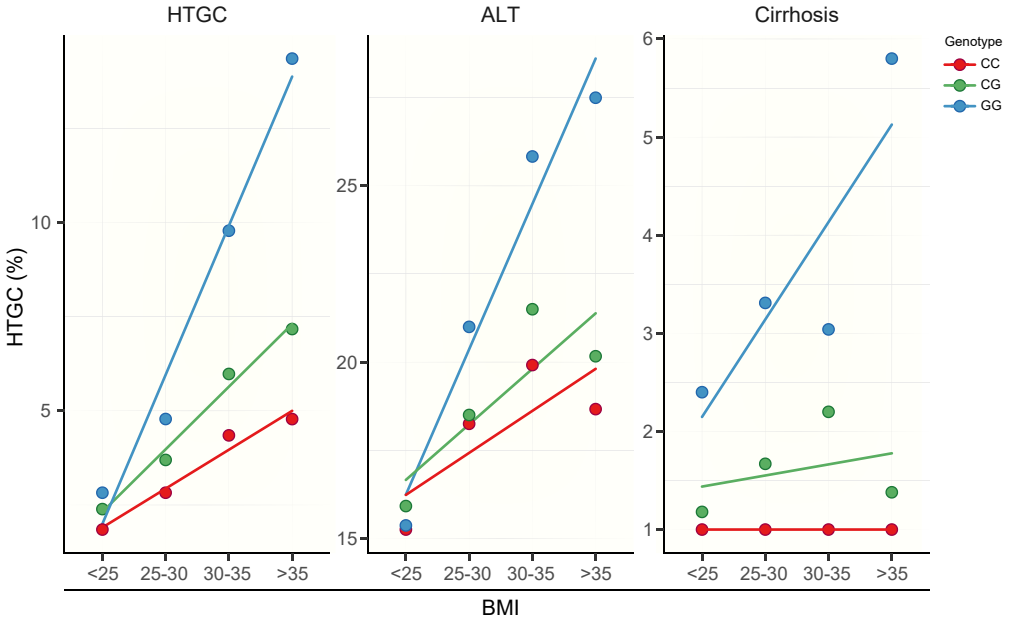

Fig. 3. Impact of BMI and *PNPLA3* (rs738409) genotype on steatosis, inflammation and cirrhosis. The figure illustrates the interaction between rs738409[G] (I148M variant) and BMI and their synergic effect on hepatic triglyceride content, serum alanine aminotransferase levels, and the risk of cirrhosis (modified from Stender *et al.*[95]). ALT, alanine aminotransferase; BMI, body mass index; HTGC, hepatic triglyceride content; OR, odds ratio; PNPLA3, patatin-like phospholipase domain-containing 3.

A roadmap to clinical translation

Based on the evidence reviewed in this manuscript, we propose a checklist for genetic studies aimed at further advancing the field of NAFLD, to ensure methodological robustness, reproducibility, and clinical relevance (Table S1). This is not an alternative to the STrengthening the REporting of Genetic Association Studies (STREGA) reporting guidelines,[97] which should be taken into consideration, but provides specific suggestions relevant for NAFLD.

Disease prediction

Results of variant associations in GWAS are usually reported with their effect sizes (*e.g.* ORs) and *p* values.[83] These metrics assess the strength of an association but do not reflect the ability of the variant to classify individuals between cases and control.[98] Thus, modest to large ORs and extreme statistical significance do not necessarily ensure clinical relevance and other measures like sensitivity, specificity, and especially positive and negative predictive values might be more appropriate for risk prediction.[99] Although the most appropriate method to evaluate the utility of genetic variant risk estimates is still under debate,[100] their performance is frequently assessed by the area under the ROC curve (AUC), which summarises the true-positive rate (sensitivity) and false-positive rate (1 – specificity) for a binary outcome, the AUC values range from 0.5 to 1 corresponding to a null and perfect predictive ability, respectively.[101] The proportion of explained heritability impacts the specificity and sensitivity of the genetic classifier tested, thus as more heritability in a phenotype is explained, the AUC will increase.[102]

In the field of FLD, the predictive value of *PNPLA3* I148M has been frequently discussed. This was justified by the robust and reproducible association with ORs often greater than 2 for various outcomes, independently of classical risk factors.[9,103] The contribution of *PNPLA3* I148M to NAFLD heritability may range from mild,[13] to as much as 5–10% of the total variation in liver fat.[95] Although remarkable, this proportion remains modest for a relevant clinical predictor, and accordingly, EASL guidelines do not yet recommend the use of this variant in routine clinical practice to assess the risk of liver damage and HCC in NAFLD.[104] Indeed, carriage of the variant had a high specificity for NAFLD-related HCC at the population level, but the accuracy was lower in a subsequent study.[26,37,105]

Since no single SNP is capable of adequate risk stratification in complex diseases, the predictive ability of gathering numerous variants in polygenic risk scores (PRSs) is a sensible approach.[100] PRSs reflect the risk aggregation of multiple variants and might be calculated as a weighted sum of disease-risk alleles carried by an individual.[106] This method has been shown to successfully pinpoint individuals at an increased risk of developing coronary heart disease (3–5-fold greater risk than the rest of the studied population),[107] and outperform existing clinical models for the prediction of breast cancer with personalised recommendations for screening.[108] Unsurprisingly, the incorporation of other risk factors into an integrative model

Key point

No single genetic variant is capable of adequate risk stratification in NAFLD. However, combining numerous variants in polygenic risk scores is an attractive approach that has shown promising results in other complex traits.

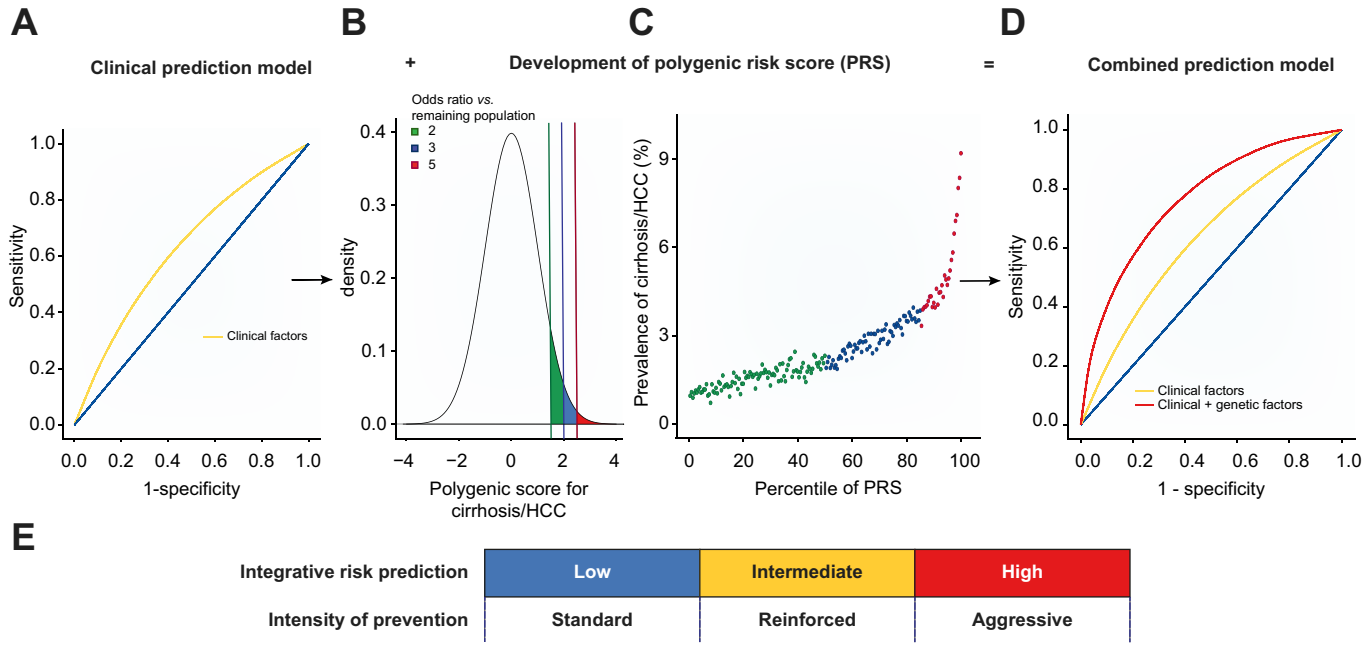

Fig. 4. Development of predictive models including clinical and genetic factors to identify at risk individuals and individualize prevention. (A) The predictive ability of a clinical model including *e.g.* age, gender, presence of diabetes and BMI for severe NAFLD. (B) The distribution (mean is set to 0 with a standard deviation of 1) of a hypothetical PRS, including *e.g. PNPLA3*, *TM6SF2*, *GCKR* and thousands of variants in the same population. The colours show the proportion of the population with an odds ratio of 2, 3, and 5 compared to all remaining patients in lower percentiles. (C) Relationship between percentile of the PRS and prevalence of cirrhosis/HCC. Again, colours highlight the odds ratio of prevalence in a given percentile *vs.* all remaining patients in lower percentiles. (D) Incorporation of genetic variants in the clinical model improves the predictive ability. (E) Risk stratification based on the aforementioned model in order to individualized preventive measures. BMI, body mass index; GCKR, glucokinase regulator; HCC, hepatocellular carcinoma; PCSK7, proprotein convertase subtilisin/kexin 7; PNPLA3, patatin-like phospholipase domain-containing 3; PRS, polygenic risk score.

significantly improves the overall risk prediction of PRS alone.[109]

Development of PRSs is emerging in the field of FLD where common and rare variants have been robustly associated with the risk of progressive NAFLD independently of clinical risk factors including the severity of liver fibrosis,[14,22,38] but there are very few data on their clinical usefulness so far. Accordingly, a comprehensive PRS was superior to evaluation of *PNPLA3* I148M and *TM6SF2* E167K alone, and led to an improvement of risk prediction in about 20% of patients with NAFLD not identified by classical risk factors in a cross-sectional study.[37] These initial figures may underestimate the utility of PRSs in disease risk stratification, as their full relevance in predicting long-term outcomes independently of the baseline severity of the disease, as determined by clinical, biochemical and imaging data, will only be appreciated when data from long-term prospective studies become available. Of note, as PRSs are derived from GWAS that have mostly been performed in individuals from European descent they may not be meaningful to populations of other ancestry.[110] Failure to include individuals from diverse ancestry will increase heath disparities and hamper the utility of genetic findings like PRSs in the multi-ethnic populations seen in clinical practice.[89]

PRSs in combination with environmental factors have the potential to improve disease screening for NAFLD-related cirrhosis and HCC and to help target lifestyle interventions to high-risk individuals (Fig. 4). Nevertheless, the utility of this integrative risk approach will be even more beneficial to clinical decision-making when more effective therapeutic interventions become available in NAFLD. However, before PRSs can be effectively translated into daily clinical practice they will need to be extensively validated in studies assessing a) clinical utility – preferably in large-scale prospective cohorts which are less prone to bias than case-control studies and where positive and negative predictive values can be directly estimated[111] – b) cost-effectiveness, and c) communication strategy to provide meaningful risk information to patients but also to hepatologists and other physicians unfamiliar with these risk metrics and important caveats.[100,110,112]

Optimisation of medical therapy and drug discovery

In keeping with the role of adiposity in triggering the phenotypic expression of the *PNPLA3* I148M variant, homozygotes have a greater reduction in liver fat following rapid weight loss than wild-type individuals.[113] The genetic background may also affect the mechanism underlying NAFLD development; for example in *PNPLA3* I148M carriers, fat accumulation seems more dependent on reduced remodelling than on increased *de novo*

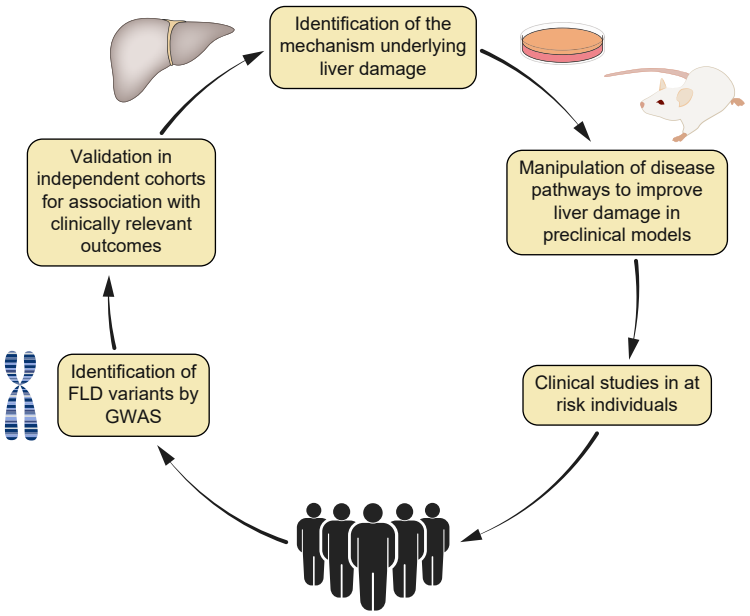

Fig. 5. A precision medicine approach to develop new NAFLD therapeutics. FLD, fatty liver disease; GWAS, genome-wide association studies; NAFLD, non-alcoholic fatty liver disease.

lipogenesis.[114] This concept may help explain independent observations that *PNPLA3* I148M carriers do not show improvement of liver damage from approaches that target hepatic lipogenesis, including supplementation with ω3 fatty acids and statin therapy, which on the other hand may be beneficial in non-carriers.[115–117]

FLD risk variants can also predict the likelihood of liver-related adverse events in response to hormones. Carriage of the *PNPLA3* I148M variant modifies the impact of basal insulin peglispro on hepatic fat accumulation, leading to a higher probability of developing liver damage in homozygous patients with type 2 diabetes shifted to this treatment.[118] These data suggest that it will be key to evaluate the influence of the genetic background on new therapeutic approaches for NASH.[119] In addition, evaluation of FLD risk variants could be implemented in clinical studies for stratification of progression risk and sub-phenotyping of NAFLD.

The available evidence shows that the selection of genetically supported drug targets doubles the likelihood of successful clinical development and may therefore improve the cost-effectiveness of the drug development pipeline.[120] Interestingly, a variant with a small effect size on protein level and disease risk does not imply that the related protein will not be a relevant drug candidate.[93] As an illustration, common SNPs near *PCSK9* – encoding a serine protease binding to low-density lipoprotein (LDL) receptors – have mild influence on LDL-cholesterol levels which contrast with the strong impact of the inhibition of PCSK9 mediated by a monoclonal antibody.[91,121]

Genetic variants are inherited at conception independently of confounding factors for NAFLD. When FLD risk variants have a robust impact on biological pathways or on the expression/activity of specific proteins, under some assumptions these can be used as lifelong proxies to gain insight into the impact of therapeutic manipulation of these specific targets. This “Mendelian randomisation” approach – a genetic epidemiology method that uses genetic variants to determine whether an observational association between a risk factor and a given phenotype is consistent with a causal effect[122] – has already led to the development of innovative therapies against cardiovascular diseases.[123] Therefore, identification of new FLD risk variants can lead to the selection of the most promising pharmacological targets to treat this condition (as recently reviewed by Romeo *et al.* in[124]). For example, when the detrimental effect of a variant is due to a new activity of the protein allele associated with FLD risk, as in the case of *PNPLA3* I148M, silencing of the expression in the liver may represent a promising approach.[125] In a proof-of-principle study, antisense oligonucleotides against *Pnpla3* were injected into mice fed steatogenic diets. These were able to reduce hepatic fat, inflammation, and fibrogenesis in mice engineered to express the Pnpla3 I148M protein more markedly than in wild-type littermates.[126] These data support the feasibility of a precision medicine approach to eliminate the cause of liver damage in carriers of specific genetic determinants (Fig. 5). Whether the utility of this personalised approach will be confirmed in clinical studies and for rarer genetic determinants of severe NAFLD[37,127] remains to be determined.

Conclusions and practical recommendations

Specific genetic risk variants have now been robustly confirmed to exert a large impact on NAFLD, with an effect size comparable and synergic to that of the main metabolic risk factors, namely obesity and type 2 diabetes. This risk increase extends to the development and progression of the full spectrum of NAFLD, extending to liver-related and overall mortality. Furthermore, genetic risk variants may be able to profile subsets of patients with different pathophysiology and response to treatment. We therefore surmise that the time has come to evaluate the efficacy and cost-effectiveness of genotyping FLD variants in clinical practice and research.

As single genetic variants are incapable of individual risk profiling, the most attractive approach to date is the development and validation of PRSs. However, the number of loci currently associated with NAFLD prevalence and outcomes remains limited compared to other complex diseases (Fig. 1). Since sample size remains the main

Table 2. Settings of application of genetic testing in NAFLD/NASH for research and clinical purposes.

Research				
Application	**Goal**	**Instrument**	**Stage of development**	**Future perspectives**
Identification of new genetic determinants	Discovery new causes of disease and the underlying mechanism	GWAS using SNP arrays followed by imputation, WES, WGS and candidate gene studies	Ongoing	Gain a more complete picture by identifying variant with a smaller effect and less frequent; Identification of new therapeutic targets
Development of PRS	Improve risk stratification	Genetic scores or algorithms	Ongoing	Develop new predictive tools based on artificial intelligence algorithms
Mendelian randomisation studies	Examine the causal relationship between NAFLD or alteration in specific pathways and clinical outcomes	Single variants and PRS	Ongoing	Identify the role of liver fat in extra-hepatic complications of the disease; predict the impact of therapeutic approaches on liver damage
Clinical trials	- Recruit patients at higher risk of progression to increase power - Stratification for genetic risk - Evaluation of outcomes in genetic subgroups	Single genetic variant relevant to the drug mechanism, PRS	Early stage, scant results reported	Wider implementation, results presentation for major subgroups (based on *PNPLA3* I148M and PRS levels status)
Clinic				
Setting	**Goal**	**Instrument**	**Stage of development**	**Future perspectives**
Liver damage screening in the population or high-risk groups	Identify patients with severe NAFLD/NASH	PRS combined with classical risk factors	Scant data available for single variants	Collect data in large cross-sectional cohorts and combine algorithms with classical risk factors, non-invasive biomarkers and imaging studies
Stratification of the risk of liver-related events	Identify patients with progressive NAFLD/NASH	PRS combined with classical risk factors	Scant data available for single variants	Collect data on prospective cohorts; utility for indication for therapy at early stage to maximize the beneficial impact?
HCC surveillance	Identify patients for whom surveillance is cost-effective	PRS combined with classical risk factors	Initial data from cross-sectional studies	Validation in multicentre perspective cohorts
Prediction of response side/effects of therapies	Personalise therapeutic managements	Single variants and PRS	Initial data for single variants for drugs for cardiometabolic risk prevention	Collect data on therapies indicated for NAFLD/NASH

GWAS, genome-wide association studies; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PRSs, polygenic risk scores; WES, whole-exome sequencing; WGS, whole-genome sequencing.

limitation, performing additional powerful GWAS using SNP arrays and next-generation sequencing methods (WES, WGS) will result in the discovery of additional common and rare variants and inevitably enhance our comprehension of NAFLD biology and individual risk stratification, aiding drug development. Therefore, we strongly advocate for the reinforcement and the creation of novel large-scale collaborative initiatives to gather and/or build extensively phenotyped cohorts with prospective follow-up in Europe and worldwide. These new GWAS will also have to include individuals regardless of ethnic background if genetic discovery and precision medicine are to benefit all patients with FLD. Although we are not ready for the translation of PRSs into daily clinical practice, the time may have come to evaluate the efficacy and cost-effectiveness of genotyping FLD variants in combination with other risk factors in (prospective) population-based cohorts.

Finally, the possible scenarios for application of genetic testing for both clinical research and practice in NAFLD/NASH in the near future are presented in Table 2.

Abbreviations

ABHD5, Abhydrolase-containing domain 5; APOB, apolipopoprotein B; AUC, area under the ROC curve; BMI, body mass index; FLD, fatty liver disease; GCKR, glucokinase regulator; GWAS, genome-wide association studies; HCC, hepatocellular carcinoma; HFE, haemochromatosis gene; HSD17B13, 17-beta hydroxysteroid dehydrogenase 13; HTGC, hepatic triglyceride content; IFNL4, interferon lambda 4; LDL, low-density lipoprotein; LIPA, lysosomal acid lipase; MARC1, mitochondrial amidoxime reducing component 1; MBOAT7, membrane bound O-acyl transferase 7; MERTK, Mer T kinase; MTTP, microsomal triglyceride transfer protein; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio; PNPLA3, patatin-like phospholipase domain-containing 3; PPP1R3B, protein phosphatase 1 regulatory subunit 3B; PRS, polygenic risk score; SNP, single nucleotide polymorphism; SOD2,

mitochondrial superoxide dismutase; TERT, human telomerase reverse transcriptase; TM6SF2, transmembrane 6 superfamily member 2; UCP2, uncoupling protein 2; VLDL, very low-density lipoprotein; WES, whole-exome sequencing; WGS, whole-genome sequencing.

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Conflict of interest

LV reports having received speaking fees from: MSD, Gilead, AlfaSigma, AbbVie; consulting fees from: Gilead, Pfizer, Astra Zeneca, Novo Nordisk, Intercept pharmaceuticals, Diatech Pharmacogenetics; unrestricted research grants from: Gilead.

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Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contribution

Authors contributed equally to the design, conceptualisation, writing of the manuscript, LV supervised the process.

Supplementary data

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Author names in bold designate shared co-first authors

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