

# The Genetics of Nonalcoholic Fatty Liver Disease: Spotlight on *PNPLA3* and *TM6SF2*

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## Abstract

Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum that spans simple steatosis, through nonalcoholic steatohepatitis (NASH) to fibrosis and ultimately cirrhosis. Nonalcoholic fatty liver disease is characterized by substantial interpatient variation in rate of progression and disease outcome: Although up to 25% of the general population are at risk of progressive disease, only a minority experience associated liver-related morbidity. Nonalcoholic fatty liver disease is considered a complex disease trait that occurs when environmental exposures act upon a susceptible polygenic background composed of multiple independent modifiers. Recent advances include the identification of *PNPLA3* as a modifier of disease outcome across the full spectrum of NAFLD from steatosis to advanced fibrosis and hepatocellular carcinoma; and the discovery of *TM6SF2* as a potential “master regulator” of metabolic syndrome outcome, determining not only risk of advanced liver disease, but also cardiovascular disease outcomes. In this article, the authors will review the field, discussing in detail the current status of research into these important genetic modifiers of NAFLD progression.

## Keywords

- ▶ nonalcoholic fatty liver disease
- ▶ *TM6SF2*
- ▶ *PNPLA3*
- ▶ gene
- ▶ steatohepatitis
- ▶ fibrosis

Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum of liver disease ranging from simple steatosis (hepatic triglyceride content [HTGC] > 5%), through steatohepatitis (fat plus inflammation and hepatocellular ballooning degeneration; NASH), to fibrosis and ultimately cirrhosis, in the absence of excessive alcohol consumption (a threshold of < 20 g/d for women and < 30 g/d for men is typically adopted).<sup>1</sup> Nonalcoholic fatty liver disease is strongly associated with features of the metabolic syndrome including abdominal obesity, insulin resistance or type 2 diabetes mellitus (T2DM) and atherogenic dyslipidaemia.<sup>2,3</sup> Over the past few decades, lifestyles have become increasingly sedentary and dietary patterns have changed, leading to an increased prevalence of obesity and insulin resistance.<sup>3</sup> Against this background, NAFLD has rapidly become the most common cause of abnormal liver biochemistry in many countries and is predicted to be the most common indication for liver transplantation within a decade.<sup>4–6</sup>

The prevalence of NAFLD and its natural history are reviewed elsewhere in this issue.<sup>7</sup> A substantial proportion of the population are at risk of progressive NAFLD due to the presence of obesity and insulin resistance. However, it is apparent that only a minority progress to more advanced disease characterized by NASH, advanced fibrosis, and hepatocellular carcinoma (HCC).<sup>3</sup> Indeed, studies in cohorts of patients that have undergone serial liver biopsies indicate that approximately 40% of NAFLD cases will develop progressive liver fibrosis while the remaining 60% exhibit stable disease or some degree of regression during long-term follow-up.<sup>8,9</sup> Rates of fibrosis progression are also nonuniform, with some individuals progressing more rapidly than others.<sup>8,9</sup> It should also be noted that liver-related mortality occurs in < 5% of NAFLD patients and is the third most common cause of death after cardiovascular disease and extrahepatic malignancy.<sup>10</sup>

The reasons for these variations remain incompletely understood, but NAFLD is best considered a complex disease

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trait where subtle interpatient variations including host genetic factors and environment interact to produce disease phenotype and determine disease progression.<sup>11–13</sup> Although the presence of NAFLD is principally determined by environmental factors, it is clear that genetic factors contribute and crucially determine how individuals respond to the challenge of caloric excess and consequent metabolic stressors. Although much work remains to be done, substantial progress in our understanding of genetic modifiers has been made. Here we will summarize the latest developments since we last reviewed the field, in particular focusing on the *PNPLA3* and *TM6SF2* genes.<sup>13,14</sup>

## Evidence Indicative of a Genetic Component to NAFLD

Susceptibility to high-prevalence diseases such as obesity, T2DM, cardiovascular disease, and NAFLD comprises a heritable component variously accounting for up to 30 to 50% of relative risk.<sup>15</sup> These complex traits result from environmental exposures acting on a susceptible polygenic background made up of multiple independent modifiers.<sup>15,16</sup> Three strands of evidence suggest that there is a significant heritable component to NAFLD: familial aggregation,<sup>17–19</sup> twin studies,<sup>20</sup> and interethnic differences in susceptibility.<sup>21–25</sup> Although socioeconomic factors can confound analysis of interethnic differences, variations in population prevalence of genetic variants such as the *PNPLA3* rs738409 polymorphism do seem to contribute to this variability.<sup>26</sup>

## Identifying Genetic Modifiers of NAFLD

The allelic frequency of susceptibility loci in common diseases remains a contentious subject.<sup>27–29</sup> Most studies are founded on the pre-eminent “common disease/common variant” (CD/CV) hypothesis.<sup>16,28</sup> Broadly, common diseases are of late onset, and so have little impact on reproductive fitness. Therefore, causative mutations are not subject to negative selection pressures, and disease susceptibility is due to the combined effects of multiple relatively common causative polymorphisms (minor allele frequency 1–5%) that are carried by affected individuals.<sup>28</sup> Discussion of the relative merits of this and the competing “multiple rare-variant” hypothesis is outside the scope of this review, but are discussed elsewhere. Although one cannot accurately predict the allelic effect-size distribution in NAFLD, it is likely that the majority of modifier loci will each individually have only a small effect, although a minority may have greater effect.<sup>28</sup> This seems consistent with evidence from genome-wide association studies (GWASs) in other conditions showing that disease-associated allelic variation is frequently noncoding and perturbs gene expression or exon splicing; subtle changes that in general have modest functional consequences compared with the much less common nonsynonymous coding sequence alterations.<sup>28,30</sup> Supporting this, the majority of published association studies report allele relative risk ratios of 1.1 to 1.5.<sup>30–32</sup>

As no single gene is sufficient to determine outcome, clear patterns of inheritance are not seen within kinships and so family-based gene linkage studies lack the necessary power to detect genetic variants as each individually confers only a modest effect.<sup>15,16</sup>

## Hypothesis-Driven Candidate-Gene Association Studies

The principal approach to the study of complex disease traits such as NAFLD has been the case-control disease-association study. These genetic association studies assess correlations between genetic variants and trait differences on a population scale and so have greater power to detect effect.<sup>33</sup> However, selection of candidate genes is founded on an assumed biologically plausible pathogenic role for the encoded protein. As these studies are reliant on there being a valid a priori hypotheses for gene selection, candidates are drawn from the limited pool of genes where biological function is already understood and considered relevant to the disease.<sup>33,34</sup> These studies are therefore unlikely to identify a role for genes that were not already implicated. As will be discussed below, candidate-gene studies have only identified a small number of genes that are robustly and reproducibly associated with NAFLD.

## Hypothesis Generation by Genome-Wide Association Studies

Patterns of association among single nucleotide polymorphisms (SNPs) throughout the genome may be characterized in terms of both linkage disequilibrium (LD; correlation between nearby variants on the same chromosome such that alleles are nonrandomly associated) and haplotype (a combination of alleles at multiple linked loci on a chromosome that are transmitted together).<sup>35</sup> The use of *tag* SNPs to serve as proxies for other SNPs that are in strong LD, allowed development of genotyping arrays that simultaneously test > 1 million different polymorphisms. These arrays permit the profiling of the majority of common (minor allele frequency [MAF] > 5%) variability in the human genome.<sup>35–37</sup>

Genome-wide association studies have led to the identification of novel genes contributing to several important diseases.<sup>34,35</sup> However, due to the nonhypothesis-driven nature of GWAS, the loci identified are frequently novel and would not otherwise have been linked to NAFLD. A corollary of this is that neither biological function nor pathogenic mechanisms are necessarily apparent and so additional study, both to confirm causality and elucidate the pathogenic mechanisms, is required. It is also apparent that a substantial proportion of disease heritability remains elusive and so GWASs are not a panacea.<sup>32</sup> The failure to achieve universal success in the study of complex diseases is in part because of the complexity of the phenotypes studied and difficulty in establishing sufficiently large, well-characterized patient cohorts. The latter is especially relevant to NAFLD where histologically characterized patients are required to assess modifiers of steatohepatitis and fibrosis. Current arrays do not yet capture rare variants with more modest effect size (MAF < 0.5%), nor are they effective at assessing disease associations with

structural polymorphisms such as copy number variants, insertions, deletions, and inversions.<sup>32,35</sup>

## GWASs by Phenotype and Chronology

At the time of writing, several genome-scale studies for NAFLD have been reported (summarized in **Table 1**).<sup>26,38–44</sup> These can be divided into three groups based on disease phenotype studied: (1) radiologically measured steatosis (HTGC), (2) histologically characterized NAFLD, and (3) clinical biochemistry phenotypes.

### Radiologically Determined Steatosis-Based GWASs

The first GWAS in NAFLD, published in 2008,<sup>26</sup> was of relatively modest size by current standards. It examined 9,229 nonsynonymous SNPs in a North American population of diverse ethnicity (Hispanic, African American, and European ancestry) from the Dallas Heart Study.<sup>26,45</sup> Hepatic triacylglycerol (TAG) accumulation was measured using non-invasive proton magnetic resonance spectroscopy (<sup>1</sup>H-MRS). This study identified a single highly significant association between increased HTGC and the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene.<sup>26</sup> The index single nucleotide polymorphism (SNP) in *PNPLA3* (rs738409 c.444 C > G, p.I148M) is a nonsynonymous cytosine to guanine nucleotide transversion mutation that results in an isoleucine to methionine amino acid change at codon 148. A gene dosage effect for I148M carriage was observed with a stepwise increase in HTGC with increasing carriage of the minor allele and homozygotes I148M carriage being associated with a twofold increase in HTGC. As alluded to earlier, minor-allele frequency correlated with ethnic differences in susceptibility to greater HTGC. I148M was most common in Hispanics (MAF 0.49), lower in those of European ancestry (0.23) and lowest in African Americans (0.17). Although less well characterized, this study also identified a second nonsynonymous SNP in *PNPLA3* (rs6006460, p.S453I) that was associated with reduced HTGC and had the opposite ethnic distribution.<sup>26,46</sup> The two SNPs in combination accounted for 72% of the ethnic variation in HTGC in the population studied.

In their later study, Speliotes et al employed a two-stage approach.<sup>39</sup> The initial exploratory meta-analysis of data from four cohorts (Age/Gene/Environment Susceptibility-Reykjavik Study, Old Order Amish Study, Family Heart Study, and Framingham Heart Study) using 2.4 million SNPs imputed to HapMap in 7,176 individuals of European ancestry remains the largest GWAS meta-analysis for NAFLD to date.<sup>39</sup> Adopting a permissive significance threshold ( $p < 10^{-3}$ ), SNPs associated with HTGC were identified and then taken forward in a separate cohort of 592 patients with histologically characterized NAFLD as a candidate gene study. Variants located in or near *PNPLA3* (rs738408), protein phosphatase 1, glucokinase regulator (*GCKR*; rs780094), regulatory (inhibitor) subunit 3B (*PPP1R3B*; rs4240624), and lysophospholipase-like 1 (*LYPLAL1*; rs12137855) were identified. In addition, a variant in the *NCAN* gene (rs2228603 c.274 C > T, p.Pro92Ser) in a region of chromosome 19 (19p13.11) was also associated with increased HTGC.<sup>39</sup> *PNPLA3*, *GCKR*, *LYPLAL1*, and *NCAN* (the

19p13.11 region), but not *PPP1R3B* were validated for histological steatosis, and additionally associated with lobular inflammation and/or fibrosis. In addition, variants in or near *NCAN*, *GCKR*, and *PPP1R3B* were associated with altered serum lipid levels and variants near *GCKR* and *PPP1R3B* affected glycemic traits. The rs738408 SNP in *PNPLA3* identified in this study is in strong LD with rs738409, previously described by Romeo et al.<sup>26</sup>

Although further study of each highlighted gene is still warranted to explore the associated pathogenic mechanisms, most were biologically plausible. *GCKR* encodes a regulator of carbohydrate metabolism and so is also highly relevant to NAFLD pathogenesis despite the precise causative variants within the gene remaining ill defined. *LYPLAL1* encodes a protein involved in triglyceride breakdown and so is likely to be relevant. A variant in *PPP1R3B* (rs2126259), which encodes the catalytic subunit of protein phosphatase-1 and so may influence hepatic glycogen synthesis, has since been validated for HTGC in another GWAS.<sup>42</sup> These contrast with the 19p13.11 locus where attention had focused on the *NCAN* gene that contained the rs2228603 SNP. Although the association was subsequently validated in a separate cohort,<sup>47</sup> *NCAN* lacked biologically plausible evidence of a functional role in NAFLD while nearby genes had previously been associated with variations in plasma triglyceride and low-density lipoprotein levels and so were more likely candidates.<sup>48,49</sup> Careful inspection of the LD patterns around rs2228603 and the 19p13.11 region suggested that the variant was in strong LD with a cluster of other SNPs up to 400 kb distant and so tagged > 20 other genes (**Fig. 1**). This led us to question whether *NCAN* was indeed the causative gene.<sup>14</sup>

Clarification on this point was provided by a subsequent GWAS based on a genome-wide exome chip in 2,736 individuals drawn from the same North American population as the original Romeo study.<sup>43</sup> In addition to again validating the association between *PNPLA3* and NAFLD, Kozlitina et al determined that a nonsynonymous genetic variant within a gene of unknown function called *TM6SF2*, *transmembrane 6 superfamily member 2* (rs58542926 c.449 C > T, p.Glu167Lys) at the 19p13.11 locus was associated with <sup>1</sup>H-MRS quantified HTGC.<sup>43</sup> Homozygote *TM6SF2* rs58542926 minor (T) allele carriage was shown to be associated with a modest, but statistically significant increase in <sup>1</sup>H-MRS measured HTGC from  $5.86 \pm 0.25\%$  in CC homozygotes to  $15.04 \pm 2.23\%$  in TT homozygotes.<sup>43</sup> This *TM6SF2* variant is in strong linkage disequilibrium with other SNPs around the 19p13.11 locus, including the rs2228603 SNP previously identified by Speliotes et al.<sup>39</sup> Crucially, conditional analyses demonstrated that *TM6SF2* rs58542926, not *NCAN* rs2228603 was the causal variant driving the association with HTGC at this locus.<sup>43,50</sup>

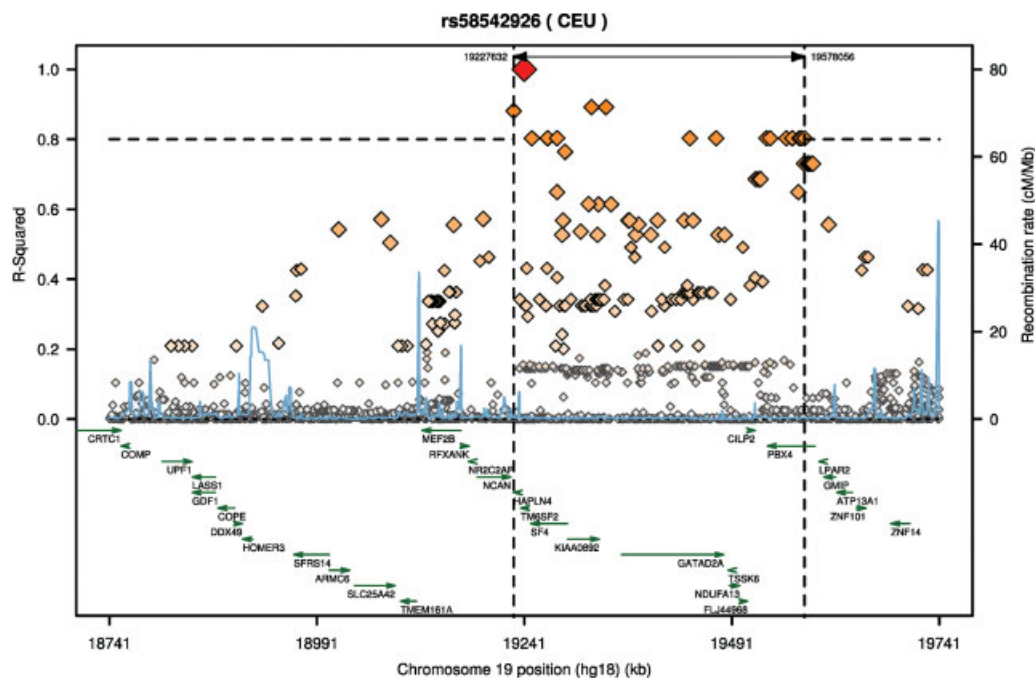
### Histologically Characterized NAFLD-Based GWASs

Reflecting the difficulty establishing large patient cohorts that are characterized using invasive tests such as liver biopsy, most histologically based GWASs published to date have used relatively small cohorts. Four histologically based GWASs have been published to date. It is notable that these have not been as productive in terms of robustly

**Table 1** Genome-wide association studies examining phenotypes relevant to nonalcoholic fatty liver disease

	Phenotype	Population	N	Number of SNPs	Modifier loci/genes identified
Romeo et al, 2008 <sup>26</sup>	<sup>1</sup> H-MRS measured steatosis	USA (mixed ethnicity)	2,051 (B: 1,032; W: 636; H: 383)	9,229	PNPLA3 (rs738409)
Yuan et al, 2008 <sup>58</sup>	Clinical biochemistry (ALT)	European (mixed ethnicity)	7,715	–	PNPLA3-SAMM50 gene cluster, CPN1-ERLIN1-CHUK gene cluster
Chalasanani et al, 2010 <sup>38</sup>	Liver histology	USA (all Female, European Caucasian)	236	324,623	FDFT1 (rs2645424) COL13A1 (rs1227756) EFCAB4B (rs887304) PZP (rs6487679)
Speliotes et al, 2011 <sup>39</sup>	CT measured Steatosis (with histological 'candidate gene' validation set)	USA & Europe (meta-analysis of previous studies)	7,176	Range 329 k–618 k before imputation	PNPLA3 (rs738409) NCAN (rs2228603) GCKR (rs780094) LYPLAL1 (rs12137855) PPP1R3B (rs4240624)
Chambers et al, 2011 <sup>59</sup>	Clinical biochemistry (ALT)	European (mixed ethnicity)	61,089	~2.6 million genotyped and imputed	PNPLA3 (rs738409) TRIB1 (rs2954021) HSD17B13 (rs6834314) CPN1 (rs10883437)
Kawaguchi et al, 2012 <sup>41</sup>	Liver histology	Japan	529	484,751	PNPLA3 (rs738409)
Anstee et al, 2012 <sup>40,131</sup>	Liver histology	European	1,125	–	PNPLA3 (rs738409)
Kitamoto et al, 2013	Liver histology	Japan	392	261,540	PNPLA3 (rs738409)
Feitosa et al, 2013 <sup>42</sup>	CT measured steatosis	USA	2,705	~2.4 million genotyped and imputed	PNPLA3 (rs738409), PPP1R3B (rs2126259), ERLIN1-CHUK-CWF19L1 gene cluster (9 SNPs in two haplotype blocks)
Kozlitina et al, 2014 <sup>43</sup>	<sup>1</sup> H-MRS measured steatosis	USA (mixed ethnicity)	2,735	247,870 exonic SNPs	PNPLA3 (rs738409), TM6SF2 (rs58542926)
DiStefano et al, 2015 <sup>44</sup>	Liver histology	USA (mixed ethnicity)	2,300	–	PNPLA3 and SUGP1 (neighboring gene to TM6SF2)

Source: Modified from Anstee QM, Day CP. The genetics of NAFLD. *Nat Rev Gastroenterol Hepatol* 2013;10(11):645–655.<sup>14</sup>  
Abbreviations: ALT, alanine transaminase; CT, computed tomography; <sup>1</sup>H-MRS, proton magnetic resonance spectroscopy; SNPs, single nucleotide polymorphisms.



**Fig. 1** Linkage disequilibrium patterns in the chromosome 19 (19p13.11) region flanking *TM6SF2*. Visualization of the 19p13.11 region flanking the rs58542926 *TM6SF2* SNP (red diamond) based on 1000 genome data analyzed in SNAP.<sup>189</sup> Strong linkage disequilibrium (LD) with a cluster of variants up to ~400 kb distant is demonstrated. Neighboring genes associated with relevant phenotypes in other studies (including *NCAN*, *SUGP1* (*SF4*), *CILP2*, *PBX4*, and *GMIP*) are likely to have been due to the LD effect across the 19p13.11 region.

identifying new genetic modifiers as the radiologically based studies.

Chalasan et al reported the first histologically based GWAS in 236 female NAFLD patients.<sup>38</sup> This study did not report associations with individual components of the histological NAFLD activity score<sup>51</sup> such as steatosis; ballooning degeneration or portal inflammation. Higher overall NAFLD activity scores were associated with a variant in the farnesyl diphosphate farnesyl transferase 1 (*FDFT1*, rs2645424), an enzyme with a role in cholesterol biosynthesis in a multivariate analysis adjusting for age, diabetes, HbA1c level, body mass index [BMI], and waist:hip ratio. Additional loci were associated with fibrosis (rs343062) and lobular inflammation (including rs1227756 in the *COL13A1* gene). *PNPLA3* was not associated with any aspect of the NAFLD phenotype in this study and the *FDFT1* association was not replicated in a subsequent study using a cohort of 340 NAFLD.<sup>52</sup> Although the associations detected in this study are potentially interesting, they require further independent validation in larger patient cohorts.

Two Japanese histologically based studies have been published.<sup>41,53</sup> The first, by Kawaguchi et al,<sup>41</sup> examined 484,751 SNPs in a cohort of 529 patients with biopsy-proven NAFLD characterized according to the Matteoni classification<sup>54</sup> and 932 population controls. The second study by Kitamoto et al<sup>53</sup> initially examined 261,540 SNPs in a cohort of 392 NAFLD patients and 934 population controls and then validated their findings in a cohort of 172 patients and 1,012 controls. Both studies confirmed the association between NAFLD and SNPs flanking the *PNPLA3* gene in this ethnically distinct population, but identified no novel signals. Another study in 2,300

obese individuals again provided strong evidence of an association with *PNPLA3* and identified a locus neighboring *TM6SF2*, both linked to a steatosis phenotype.<sup>44</sup>

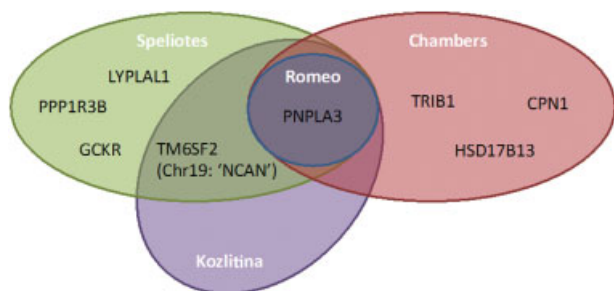
### Clinical Chemistry-Based GWASs

It is accepted that liver biochemistry correlates poorly with the presence of NAFLD or disease severity<sup>55,56</sup> so that biochemical screening consistently reports a lower disease prevalence (3–12%) than imaging or histology-based studies.<sup>57</sup> Accepting these limitations, two GWASs have identified genes that influence plasma levels of alanine transaminase (ALT).<sup>58,59</sup> The first of these studies found that *PNPLA3* I148M was associated with raised serum ALT levels in populations of European and Indian-Asian descent.<sup>58</sup> A region spanning three genes on chromosome 10, *APN1-ERLIN1-CHUK* (rs11597390, rs11591741, rs11597086) that was also highlighted in this study<sup>58</sup> has since been associated with radiological steatosis and ALT levels in a GWAS-correlated meta-analysis, which flagged variants in *ERLIN1-CHUK-CWF19L1*.<sup>42</sup> The second of the two studies identified 42 loci at genome-wide significance levels ( $p < 1 \times 10^{-8}$ ) in a cohort of 61,089 individuals.<sup>59</sup> Four loci were associated with elevations in ALT: rs6834314 near *HSD17B13* and *MAPK10*; rs2954021 near *TRIB1* (a gene that has previously been associated with variations in plasma cholesterol and triglyceride levels<sup>48,60,61</sup>); rs10883437 near *CPN1*; and *PNPLA3* rs738409.<sup>59</sup>

### The Chromosome 22 Locus: *PNPLA3*

Across all GWASs it is noteworthy that only two genes (*PNPLA3* and *TM6SF2*) have been identified as potential





**Fig. 2** Commonality between the largest genome-wide association studies (GWASs) in nonalcoholic fatty liver disease (NAFLD). There is surprisingly little commonality in genes identified in GWASs examining aspects of the NAFLD phenotype. Currently, only two genes (*PNPLA3* and *TM6SF2*) have been identified in more than one study.

modifiers in more than one study (► **Fig. 2**). Following its association with steatosis in the first reported NAFLD GWAS, there has remained considerable interest in the role of the *PNPLA3* gene on chromosome 22 as a modifier of NAFLD pathogenesis. This association has now been independently replicated in numerous candidate-gene studies examining both adult<sup>62–66</sup> and pediatric<sup>66–69</sup> NAFLD cohorts across differing ethnicities, as has the association with raised ALT/aspartate aminotransferase (AST) levels.<sup>70</sup> There is also evidence from histologically based studies that the rs738409 (I148M) variant is associated with severity of steatohepatitis and greater fibrosis.<sup>62,64,66</sup> It has also been associated with greater response to dietary or lifestyle modification and so may be a marker of both greater risk and also greater benefit from intervention.<sup>71,72</sup>

The rise in the burden of NAFLD coincides with a marked increase in the incidence of HCC in many countries,<sup>73–76</sup> and so it is timely to also consider whether genetic modifiers such as *PNPLA3* may also increase the risk of NAFLD-associated HCC. The I148M variant has been associated with increased HCC risk in a mixed-etiology cohort,<sup>77</sup> the morbidly obese,<sup>78</sup> alcohol-related liver disease,<sup>79–82</sup> and more variably, in chronic viral hepatitis.<sup>79,80,82–84</sup> This association is supported by a meta-analysis<sup>85</sup> but is best demonstrated by a recent European study, where in a multivariate analysis adjusted for age, gender, diabetes, BMI, and presence of cirrhosis, carriage of each copy of the rs738409 minor (G) allele conferred an additive risk for HCC (adjusted odds ratio [OR] = 2.26, 95% confidence interval [CI] = 1.23–4.14,  $p = 0.0082$ ), with GG homozygotes exhibiting a fivefold (adjusted OR = 5.05, 95% CI = 1.47–17.29,  $p = 0.01$ ) increased risk over CC homozygotes.<sup>86</sup> In this study, comparison of genotype frequencies between the NAFLD-HCC cohort and an unselected United Kingdom (UK) general population sample (the MRC/Wellcome Trust UK 1958 Birth Cohort) identified a 12-fold increased risk of HCC (OR = 12.19, 95% CI = 6.89–21.58,  $p < 0.0001$ ) for rs738409 minor (G) allele homozygotes relative to C-allele homozygotes.<sup>86</sup> These findings have led some to suggest that *PNPLA3*-associated HCC approaches monogenic inheritance.<sup>87</sup> We would caution against a move toward considering “*PNPLA3*-associated NAFLD,” or by extension “*PNPLA3*-associated HCC,” as distinct, monogenic conditions,

but based on a reanalysis of the data from Liu et al<sup>86</sup> to assess sensitivity/specificity agree that there may be value in the *PNPLA3* genotyping to select out those individuals least likely to develop HCC and therefore least likely to benefit from surveillance given the very high negative predictive value.<sup>88</sup> Further studies to determine the utility and health-economic merits of a multifactorial risk stratification that incorporates *PNPLA3* rs738409 genotype along with other recognized risk factors for HCC are warranted.

### The Challenge of Determining the Mechanistic Effects of *PNPLA3* Variants

The *PNPLA3* gene encodes a 481 amino acid protein that is structurally related to the principal TG hydrolase in adipose tissue, adipose triglyceride lipase (*ATGL/PNPLA2*).<sup>46,89</sup> *PNPLA3* differs from classical lipases in possessing a catalytic dyad (S47/D166) rather than the more usual triad.<sup>90</sup> Although the site of the I148M variant does not lie within this highly conserved catalytic site, the I148M amino acid substitution affects the hydrophobic substrate-binding groove and so may prevent substrate access to the catalytic site.<sup>91</sup>

It is accepted that the modifier effect of *PNPLA3* on the degree of hepatic steatosis is not due to a direct change in insulin sensitivity when assessed either by hyperinsulinemic, euglycemic clamp<sup>63,65</sup> or plasma insulin response to oral glucose tolerance testing.<sup>92</sup> Furthermore, it does not alter the overall severity of the metabolic syndrome or confer a greater BMI, degree of dyslipidemia or prevalence of overt T2DM.<sup>93</sup> Current evidence suggests that the effects are more subtle, and that carriage of the *PNPLA3* variation sensitizes the liver to metabolic stress due to nutritional calorific excess and adiposity, which is consistent with the concept of NASH as a complex disease trait. Thus, carriage of the I148M variant is associated with a greater degree of steatosis for any given degree of insulin resistance or adiposity. This concept is supported by the fact that I148M carriage is more strongly associated with raised AST/ALT in the presence of obesity.<sup>92</sup>

Despite the strong genetic evidence, the physiological role of *PNPLA3* and how this is perturbed by carriage of the I148M polymorphism is still incompletely understood. The structural changes affecting the catalytic site described above are consistent with the results of studies examining recombinant adiponutrin expressed in HUH-7 cells and the in vitro biochemical actions of purified *PNPLA3*.<sup>91,94</sup> These demonstrate that *PNPLA3* hydrolyses acylglycerols with maximal hydrolytic activity observed against the three major glycerolipids, triacylglycerol (TG), diacylglycerol, and monoacylglycerol, with a strong preference for oleic acid as the acyl moiety; and that the I148M polymorphism is associated with substantially reduced enzymatic activity without reducing substrate affinity.<sup>91,94</sup> Stable isotope tracer studies in 55 overweight/obese men and in vitro studies using rat McA-RH7777 cells that secrete ApoB-containing very-low-density lipoprotein- (VLDL-) like particles demonstrate that carriage of the I148M variant reduces VLDL secretion, an effect that may be indicative of a failure to mobilize TG from intracellular lipid droplets due to loss of TG hydrolase activity.<sup>95</sup> However, this is contentious as other groups have proposed that

*PNPLA3* may also possess lysophosphatidic acid acetyltransferase (LPAAT) activity that is enhanced by I148M, increasing TG synthesis.<sup>96,97</sup>

Interspecies differences in gene expression pattern have complicated in vivo mechanistic studies. *PNPLA3* is expressed primarily in liver and adipose tissue, where it partitions to membranes and lipid droplets.<sup>91</sup> However, hepatic expression is highest in man,<sup>98,99</sup> whereas adipose tissue expression is highest in mice.<sup>100,101</sup> Moreover, the two species only share approximately 68% *PNPLA3* homology. Hepatic *PNPLA3* expression is increased after feeding, reduced by fasting,<sup>46,101</sup> and is also raised in obesity.<sup>63,101</sup> Insulin controls postprandial *PNPLA3* expression through LXR/RXR and the transcription factor SREBP-1c.<sup>98</sup> Significantly, additional posttranslational control influenced by the constituents of the fatty acid milieu has also been described.<sup>98</sup> The presence of specific saturated (palmitate, C16:0), monounsaturated (oleate, C18:1) and polyunsaturated fatty acids (linoleic acid, C18:2) increased adiponutrin expression although very long chain fatty acids (e.g., arachidonic acid, C20:4 and eicosapentaenoic acid, C20:5) were not found to effect expression.<sup>98</sup>

Reconciling the apparently conflicting findings of the in vivo experiments with the in vitro studies has proved challenging (► **Table 2**). Although the initial in vitro data was most consistent with a loss-of-function effect of the I148M polymorphism, deletion of *Pnpla3* did not provoke hepatic TG accumulation, even when mice were fed a high-sucrose diet.<sup>102,103</sup> Adenoviral mediated overexpression of wild-type human *PNPLA3* was also unable to provoke steatosis; however, overexpression of the *PNPLA3* I148M variant did cause steatosis.<sup>91</sup> More recently, studies have shown that neither hepatic nor adipose tissue overexpression of wild-type *PNPLA3* promotes hepatic TG accumulation. Hepatic overexpression of the I148M variant form did, however, cause TG accumulation.<sup>104</sup> Importantly, three distinct metabolic effects were observed: increased synthesis of fatty acids and TG, impaired TG hydrolysis, and relative depletion of TG long chain polyunsaturated fatty acids.<sup>104</sup> Taken together, these data support the view that *PNPLA3* acts to remodel TG in lipid droplets as they accumulate in the fed-state and that I148M affects multiple facets of this process. Furthermore, the apparent mixed enzymatic actions, coupled with the transcriptional regulation of *PNPLA3* by feeding and the specific fatty acid profile suggests that the action of *PNPLA3* may vary somewhat according to tissue and metabolic milieu. It is noteworthy that in this study hepatic overexpression of the I148M variant led to increased hepatic TG, but no associated changes in TNF $\alpha$ ,  $\alpha$ -smooth muscle actin or collagen type 1 $\alpha$  mRNA expression were seen, providing no evidence that either inflammatory or fibrotic processes were active.<sup>104</sup>

In an attempt to address the concerns that overexpression of human *PNPLA3* I148M in mice may not have physiological fidelity, a recent study by Smagris et al used homologous recombination to introduce the *Pnpla3* I148M variant (or a complete loss of function mutation that distorted the catalytic dyad, S47A) in mice while preserving normal physiological regulation of gene expression.<sup>105</sup> Consistent with the concept that the I148M variant sensitizes the liver to metabolic stress

due to nutritional caloric excess and adiposity, a two to threefold increase in hepatic TG accumulation was seen when mice were fed high sucrose diet (but not standard chow), although these effects were not associated with an increase in ALT or circulating lipid levels.<sup>105</sup> Interestingly, *Pnpla3* I148M was not associated with any changes in hepatic *Srebp-1c* expression or the expression profiles of its target genes, and once again no changes in TNF $\alpha$ ,  $\alpha$ -smooth muscle actin, or collagen type 1 $\alpha$  mRNA expression were observed. A novel finding in this study was that hepatic TG accumulation in the knockin mice was accompanied by a 40-fold increase in either catalytically inactive *PNPLA3* variant (I148M or S47A) on the surface of hepatic lipid droplets. This study is of significance as it reconciles the apparent disparity between the in vitro data suggesting a predominant loss-of-function effect of I148M<sup>91,94,95</sup> and the in vivo data where *Pnpla3* knockout mice did not develop a NAFLD phenotype.<sup>102,103</sup> The I148M variant confers a dominant-negative effect and so for the variant to drive NAFLD pathogenesis the presence of catalytically inactive *PNPLA3* protein is necessary, not simply a complete absence of *PNPLA3* activity.<sup>105</sup>

While representing a substantial advance in our understanding of disease pathogenesis, the work by Li et al<sup>104</sup> and Smagris et al<sup>105</sup> underline the fact that further research is required to clarify how *PNPLA3* drives not-only hepatic lipid accumulation, but also NAFLD progression to steatohepatitis, fibrosis, and hepatocellular carcinoma, where no clear mechanisms have been identified as yet. Addressing this point, only one study has offered any evidence of mechanistic link between *PNPLA3* activity and hepatic fibrosis.<sup>106</sup> In that study, Pirazzi et al demonstrated that *PNPLA3* is highly expressed in human hepatic stellate cells (HSCs) that purified wild-type *PNPLA3* hydrolyzes retinyl palmitate into retinol and palmitic acid and that this enzymatic activity is markedly reduced by the I148M variant.<sup>106</sup> These findings suggest a potential link between HSCs, retinoid metabolism, and *PNPLA3* in determining the susceptibility to hepatic fibrosis although no evidence that the observed effects altered HSC activation was provided and so further study is needed before this mechanism can be established as relevant.

## The Chromosome 19 Locus: *TM6SF2*

The controversy regarding the causative gene within the 19p13.11 locus that was initially ascribed to *NCAN* has now largely been resolved. Kozlitina et al demonstrated that a non-synonymous genetic variant within *TM6SF2* (rs58542926 c.449 C > T, p.Glu167Lys) was associated with <sup>1</sup>H-MRS quantified HTGC.<sup>43</sup> This SNP lies within 50 kb of the *NCAN* gene variant (rs2228603) that had previously been associated with HTGC.<sup>39,47</sup> Although both SNPs are in strong linkage disequilibrium ( $D' = 0.926$ ,  $r^2 = 0.798$ ), conditioning on the *TM6SF2* variant abrogated the effect of the *NCAN* variant while the reverse did not occur, establishing that *TM6SF2* rs58542926 is more strongly associated with the HTGC phenotype.<sup>43</sup> In vitro and in vivo functional studies have provided further support for this conclusion, but did not provide evidence demonstrating whether the effect of *TM6SF2* was limited to hepatic steatosis or

**Table 2** Summary of studies examining the physiological role of PNPLA3 and the I148M variant

Study	In vivo/In vitro	Experimental conditions	Findings
Chen et al, 2010 <sup>102</sup>	In vivo	Global targeted PNPLA3 knockout mouse.	Loss of PNPLA3 (knockout) does not increase hepatic TAG accumulation across a range of dietary and genetic backgrounds.
He et al, 2010 <sup>91</sup>	Both	In vivo: adenoviral overexpression of wild type and I148M in C57BL/6J mice. In vitro: (1) recombinant PNPLA3 purified from Sf9 cells; (2) overexpression of wild type and I148M in HuH-7 cells.	PNPLA3 has hydrolase activity. I148M a loss of function mutation, reducing TAG and DAG hydrolysis.
Basantani et al, 2011 <sup>103</sup>	In vivo	Global targeted PNPLA3 knockout mouse.	Loss of PNPLA3 (knockout) does not increase hepatic TAG accumulation. Argues against I148M being a loss-of-function mutation.
Huang et al, 2011 <sup>94</sup>	In vitro	Purified human wild type and I148M	PNPLA3 has hydrolase activity. I148M a loss of function mutation, reducing TAG hydrolysis.
Kumari et al, 2012 <sup>97</sup>	Both	In vivo: Wild type and PNPLA3 knockout mice fed chow or high sucrose diets. In vitro: (1) enzymatic activity of purified murine and human wild type and I148M protein; (2) overexpression of wild type and I148M PNPLA3 in HepG2, CHO, and Cos-7 cells.	Loss of PNPLA3 (knockout) does not increase hepatic TAG accumulation. PNPLA3 has lysophosphatidic acid acetyltransferase activity (LPAAT). I148M a gain of function mutation, increasing TAG synthesis.
Perttola et al, 2012 <sup>132</sup>	In vitro	Immortalized human hepatocytes and HuH-7 cells	PNPLA3 I148M increases hepatic TAG accumulation by reducing hydrolysis.
Pirazzi et al, 2012 <sup>95</sup>	Both	In vivo: Tracer studies in patients of known PNPLA3 genotype In vitro: Targeted overexpression of I148M in McA-RH 7777 cells.	Carriage (or overexpression) of I148M associated with reduced VLDL (ApoB) secretion and thus TAG accumulation. Proposed mechanism: reduced TAG hydrolysis leading to impaired lipidation on VLDL and so reduced TAG efflux.
Li et al, 2012 <sup>104</sup>	In vivo	Targeted overexpression of wild type and I148M PNPLA3 in mouse adipose tissue and hepatocytes.	No effect of wild-type PNPLA3 overexpression in liver or adipose tissue No effect of I148M overexpression in adipose tissue Hepatocyte I148M overexpression caused hepatic TAG accumulation due to increased FFA and TAG synthesis, impaired TAG hydrolysis, and relative depletion of TAG long-chain polyunsaturated fatty acids.

(Continued)



Table 2 (Continued)

Study	In vivo/In vitro	Experimental conditions	Findings
Pirazzi et al, 2014 <sup>106</sup>	In vitro	Analysis of effects of PNPLA3 on retinol metabolism in HSCs	PNPLA3 is highly expressed in human HSCs. PNPLA3 hydrolyzes retinyl palmitate into retinol and palmitic acid. This enzymatic activity is markedly reduced by the I148M variant.
Smagris et al, 2015 <sup>105</sup>	In vivo	Knockin of I148M variant in mouse <i>Pnpl3</i> gene	No effect of I148M carriage on normal chow, increased steatosis on high sucrose diet. Variant I148M PNPLA3 is a loss of function variant that coats lipid droplets.

Source: Modified from Anstee QM, Day CP. The genetics of NAFLD. *Nat Rev Gastroenterol Hepatol* 2013;10(11):645–655.<sup>14</sup>

Abbreviations: FFA, free fatty acid; HSCs, hepatic stellate cells; LD, linkage disequilibrium; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; TAG, triacylglycerol; VLDL, very low density lipoprotein.

whether it had broader clinical relevance, modifying the risk of steatohepatitis or fibrosis as has been shown for *PNPLA3*.<sup>43</sup> Candidate gene studies addressed the effect of the *TM6SF2* rs58542926 variant on presence of steatohepatitis and severity of NAFLD-associated hepatic fibrosis/cirrhosis. The first such study, in a cohort of > 1,000 histologically characterized European Caucasian patients demonstrated that carriage of each copy of the E167K variant was associated with a near twofold increased risk of advanced fibrosis (OR = 1.88 [1.41–2.5] per copy of the minor allele carried), independent of confounding factors including age, diabetes, obesity, or *PNPLA3* genotype.<sup>50</sup> This association has since been independently validated in another large European cohort.<sup>107</sup> Two studies, including one from China, have failed to replicate the association,<sup>108,109</sup> although an association with NAFLD has been demonstrated in another Chinese population.<sup>110</sup> These two negative results may in part be due to the generally low minor allele frequency of *TM6SF2* rs58542926 and interethnic variations in its carriage. A minor allele frequency of 0.07 in Europeans, 0.04 in Hispanics, and 0.02 in African Americans has been reported,<sup>111</sup> meaning that the effect of the NAFLD promoting *TM6SF2* allele will be more apparent in individuals of European ancestry than Hispanic or African ancestry. Inadequate statistical power to detect an effect on histological markers of disease progression coupled with a cohort exhibiting predominantly mild disease were contributory flaws in the small South American study.<sup>109</sup> This study contained only 226 NAFLD cases with histologically characterized disease of which 96 had simple steatosis and the remaining 130 exhibited only minimally active disease with little fibrosis (mean fibrosis stage of 1.4 out of 4). Conclusions drawn from this low quality study are questionable and are likely to represent type 2 error. Thus, the modifier role exerted by *TM6SF2* rs58542926 (E167K) at the 19p13.11 locus has been robustly demonstrated across several large independent cohorts for NAFLD phenotypes ranging from steatosis to advanced fibrosis/cirrhosis.<sup>39,43,50,107,110,112</sup>

### The Mechanistic Effects of *TM6SF2* Variants

Little is known of the precise protein structure or functional role of the *TM6SF2* gene product beyond that it is a multipass membrane protein.<sup>113</sup> Based on the analysis of coexpressed gene profiles in the Mouse Genome Informatics (MGI) database, *TM6SF2* is predicted to function as a lipid transporter and may interact with proteins involved in intestinal absorption.<sup>114</sup> The *TM6SF2* rs58542926 (E167K) missense mutation maps to an exposed nontransmembrane domain. Its effects appear to extend beyond liver disease alone. Using confocal microscopy, investigators observed localization of GFP-tagged *TM6SF2* to the endoplasmic reticulum and ER-Golgi intermediate compartments (ERGIC) in two human hepatoma cell lines.<sup>112</sup> In a series of elegant in vitro experiments they also found that knockdown of *TM6SF2* reduced secretion of triglyceride-rich lipoproteins and APOB. This led to increased cellular triglyceride accumulation, which at the subcellular level, manifested as a marked increase in lipid droplet number and average size. Conversely, overexpression of *TM6SF2* caused a decrease in the number and average size of lipid droplets.<sup>112</sup>

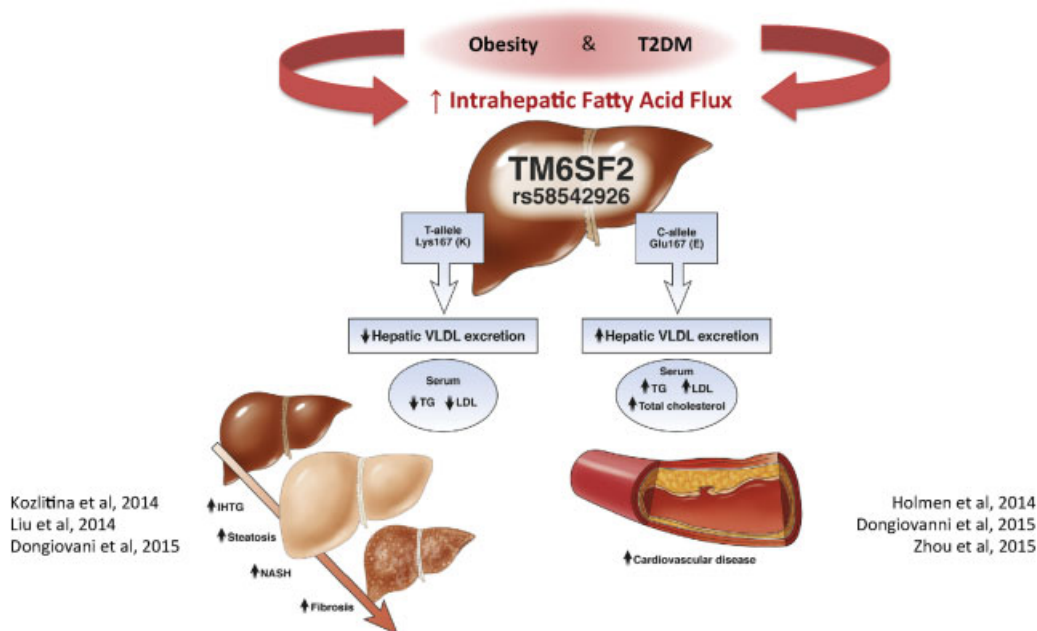
A GWAS seeking modifiers of serum lipid levels and cardiovascular disease risk published around the same time as the studies linking *TM6SF2* E167K with NAFLD supports these findings.<sup>115</sup> Holmen et al provided evidence that carriage of the more common *TM6SF2* rs58542926 major allele was associated with dyslipidemia (raised serum LDL cholesterol and triglyceride) and increased myocardial infarction/cardiovascular disease risk while minor allele carriage was protective.<sup>115</sup> This finding was also consistent with several earlier reports regarding the 19p13.11 locus that had variously implicated the nearby genes *NCAN*, *PBX4*, and *GMIP* with dyslipidemia, associations that may probably now be ascribed to *TM6SF2*.<sup>39,48,49</sup> The association of *TM6SF2* with dyslipidemia and atherosclerosis risk in humans was validated by another study examining two separate groups (a NAFLD patient cohort and an obese population cohort). Patients with NAFLD that carried the E167K variant encoding the minor allele were found to have a lower risk of developing carotid plaques (OR = 0.49, 95% CI = 0.25–0.94) while E167K carriers among a group of 1,819 obese individuals had significantly lower serum lipid levels and a lower incidence of cardiovascular events (hazard ratio = 0.61; 95% CI = 0.39–0.95).<sup>107</sup> As demonstrated in a study examining 300 Finnish subjects, the effects of *TM6SF2* on HTGC and circulating lipid profiles are mediated without inducing greater hepatic insulin resistance.<sup>116</sup> Indeed, the effects of insulin on glucose production and lipolysis were significantly higher in carriers of the *TM6SF2* E167K variant than in wild-type subjects.<sup>116</sup> The most recent GWAS, addressing the impact of low-frequency and rare variants on lipid levels, examined 9.6 million genetic variants achieved through 1,000 Genomes Project imputation in 62,166 samples.<sup>114</sup> Here, two variants within *TM6SF2* were identified that together explained the entire 19p13.11 regional association with

lipid levels (TC, LDL-C, and TG). These variants were rs58542926 (E167K) and a second, less common, missense mutation located in the fifth transmembrane domain (rs1874290064, L156P).<sup>114</sup>

Further support for the parallel effects of *TM6SF2* on NAFLD pathogenesis and hepatic lipid handling is provided by a series of in vivo studies observing the effects of altered gene expression in mice. Overexpression of human wild-type *TM6SF2* in liver resulted in higher total cholesterol (TC), low-density lipoprotein (LDL) cholesterol, triglycerides (TGs), and lower high-density lipoprotein (HDL) cholesterol, whereas knockdown of mouse *Tm6sf2* in liver resulted in decreased serum TC.<sup>115</sup> Knock down of mouse *Tm6sf2* in vivo also resulted in increased hepatic lipid accumulation and a reduction in serum TG, LDL, and VLDL secretion.<sup>43</sup> Taken together, these in vivo<sup>43,115</sup> and in vitro<sup>43,112,115</sup> functional studies suggest that *TM6SF2* controls hepatic lipid efflux, with its deletion or mutation resulting in a reduction in lipoprotein secretion (VLDL, TG, and APOB) coincident with increased hepatocellular lipid droplet size and TG accumulation. Because the E167K variant results in lower TC in humans and expression levels of the E167K protein is lower than the wild-type form, these data further indicate that the E167K variant confers a loss of function to the protein that may be both qualitative and quantitative.<sup>43,115</sup>

### ***TM6SF2* and Cardiovascular Disease: The “Catch-22” Paradigm**

Based on available evidence, we recently described the “*TM6SF2* Catch-22” paradigm.<sup>117</sup> Here, on a background of insulin resistance and metabolic stress, *TM6SF2* acts as a determinant of metabolic syndrome-related end-organ damage and thus clinical outcome: protecting the liver at the



**Fig. 3** The “*TM6SF2* Catch-22.” On a background of insulin resistance and metabolic stress, *TM6SF2* acts as a determinant of metabolic syndrome related end-organ damage and thus clinical outcome: Protecting the liver at the expense of increased risk of atherosclerosis and cardiovascular disease or vice versa. LDL, low-density lipoprotein; T2DM, type 2 diabetes mellitus; TG, triglyceride; VLDL, very low-density lipoprotein. (Modified from Kahali B, Liu YL, Daly AK, Day CP, Anstee QM, Speliotes EK. *TM6SF2*: catch-22 in the fight against nonalcoholic fatty liver disease and cardiovascular disease? *Gastroenterology* 2015;148(4):679–684<sup>117</sup> with permission.)

**Table 3** Summary of genetic modifiers of nonalcoholic fatty liver disease

Gene	Protein	Study details and comments
Glucose metabolism and insulin resistance		
ENPP1; IRS1	Ectonucleotide pyrophosphatase/phosphodiesterase family member 1; Insulin receptor substrate 1	Functional variants in <i>ENPP1/PC-1</i> and <i>IRS1</i> impair insulin receptor signaling and promote insulin resistance. <sup>133,134</sup> In 702 biopsy-proven NAFLD cases, carriage of nonsynonymous SNPs in <i>ENPP1</i> (rs1044498, encoding Lys121Gln) and <i>IRS1</i> (rs1801278, encoding Gln972Arg) reduced AKT activation, promoted insulin resistance, and were independently associated with greater fibrosis. <sup>135</sup>
GCKR	Glucokinase regulatory protein	<i>GCKR</i> SNP rs780094 is in strong LD with a functional nonsynonymous SNP (rs1260326, encoding Pro446Leu) and has been associated with hepatic TG accumulation in several studies. <sup>39,59</sup> It is also associated with greater NAFLD fibrosis. <sup>136</sup>
SLC2A1	Solute carrier family 2, facilitated glucose transporter member 1	A study examining 3,072 SNPs across 92 candidate genes identified variants in <i>SLC2A1</i> associated with NAFLD, independent of insulin resistance or T2DM. <sup>137</sup> Downregulation of <i>SLC2A1</i> in vitro promoted lipid accumulation and increased oxidative stress, potentially linking the key pathogenic features of NAFLD: oxidative injury and increased lipid storage.
TCF7L2	Transcription factor 7-like 2	A role for <i>TCF7L2</i> , which has a key role in Wnt signaling and has been implicated in T2DM, has been reported in NAFLD. <sup>138</sup>
PPARG	Peroxisome proliferator-activated receptor $\gamma$	A loss-of-function SNP (rs1805192, encoding Pro12Ala) impairs transcriptional activation and affects insulin sensitivity. <sup>139</sup> Carriage of haplotypes including the Pro12Ala allele was associated with progressive NAFLD, <sup>140</sup> but two studies found no association. <sup>141,142</sup>
Steatosis		
Hepatic lipid import or synthesis		
SLC27A5	Very long chain acyl-CoA synthetase	Two principal isoforms of FATPs are expressed in the liver, <i>SLC27A2</i> (also known as <i>FATP2</i> ) and <i>SLC27A5</i> (also known as <i>FATP5</i> ). <sup>143</sup> Silencing <i>SLC27a5</i> reverses diet-induced NAFLD and improves hyperglycemia in mice. <sup>144</sup> Carriage of the <i>SLC27A5</i> rs56225452 promoter region polymorphism has been associated with higher ALT levels, and greater postprandial insulin and triglyceride levels. <sup>144</sup> In patients with histologically proven NAFLD, the effect of BMI on degree of steatosis differed with <i>SLC27A5</i> genotype. <sup>145</sup>
FADS1	Fatty acid desaturase 1	Alleles associated with decreased hepatic expression of <i>FADS1</i> were associated with greater HTGC. <sup>128</sup>
LPIN1	Phosphatidate phosphatase LPIN1	<i>LPIN1</i> is required for adipogenesis and the normal metabolic flux between adipose tissue and liver, where it also acts as an inducible transcriptional

Table 3 (Continued)

Gene	Protein	Study details and comments
		coactivator to regulate fatty acid metabolism. <sup>146,147</sup> Variants have been associated with multiple components of the metabolic syndrome. <sup>146,148</sup> Although a large case-control study found no association with T2DM, obesity, or related traits in 17,538 individuals, <sup>149</sup> a meta-analysis in 8,504 individuals found that the <i>LPIN1</i> rs13412852 [T] allele was associated with lower BMI and insulin levels. <sup>150</sup> This same polymorphism was underrepresented in pediatric (but not adult) NAFLD with a suggestion of less severe liver damage. <sup>151</sup>
Hepatic lipid export or oxidation in steatosis		
<i>PNPLA3</i>	Patatin-like phospholipase domain-containing 3	The nonsynonymous 617C > G nucleotide transversion mutation SNP (rs738409, encoding Ile148Met) has been consistently associated with steatosis, steatohepatitis, and hepatic fibrosis; however, function remains incompletely understood. <sup>26,62</sup>
<i>TM6SF2</i>	Transmembrane 6 super family 2	The <i>TM6SF2</i> rs58542926 minor allele is associated with greater steatosis, steatohepatitis, and NAFLD fibrosis. The major allele is associated with dyslipidemia and greater CVD risk. <sup>43,50,107,115</sup>
<i>NR1H2</i>	Nuclear receptor subfamily 1 group 1 member 2 (also known as pregnane X receptor)	<i>NR1H2</i> encodes a transcription factor that regulates hepatic detoxification <sup>152</sup> and acts through CD36 (fatty-acid translocase) and various lipogenic enzymes to control lipid metabolism. <sup>153</sup> <i>Nr1h2</i> -deficient mice develop steatosis. <sup>153</sup> Two SNPs (rs7643645 and rs2461823) were associated with NAFLD and were also a predictor of disease severity. <sup>154</sup>
<i>PPARα</i>	Peroxisome proliferator-activated receptor α	<i>PPAR-α</i> is a molecular sensor for long chain fatty acids, eicosanoids, and fibrates <sup>155</sup> ; activated by increased hepatocyte fatty-acid load, it limits TAG accumulation by increasing fatty acid oxidation Carriage of a nonsynonymous SNP (rs1800234, encoding Val27Ala) increases activity, and was associated with NAFLD despite reduced BMI. <sup>156,157</sup> A loss-of-function polymorphism (rs1800206, encoding Leu162Val) was not associated with NAFLD. <sup>142</sup>
<i>PEMT</i>	Phosphatidylethanolamine N-methyltransferase	Two studies have reported an association between NAFLD and a nonsynonymous <i>PEMT</i> exon 8 590G > A transversion (rs7946, encoding Val175Met). <sup>126,127</sup>
<i>MTTP</i>	Microsome triglyceride transfer protein large subunit	<i>MTTP</i> mediates hepatic synthesis and secretion of VLDL. Abetalipoproteinemia (OMIM#200100) results from a loss-of-function frameshift mutation in <i>MTTP</i> ; however, whereas this mutation causes severe hepatic TAG accumulation, steatohepatitis and fibrosis are infrequent. <sup>158</sup> A promoter region transversion (-493G > T; rs1800591), predisposed to steatosis and NASH <sup>124,159</sup> in a small cohort, but a larger study in 131 patients found no association. <sup>160</sup>

(Continued)



Table 3 (Continued)

Gene	Protein	Study details and comments
APOC3	Apolipoprotein CIII	Two promoter region SNPs -455T > C, rs2854116 and -482C > T; rs2854117) that increased steatosis were reported in small (n = 95 and 163) cohorts of Asian-Indian and non-Asian ethnicity. <sup>161</sup> To date, studies together examining > 4,000 individuals have been unable to replicate these findings. <sup>162-165</sup>
APOE	Apolipoprotein E	ApoE is a plasma protein involved in lipid transport and metabolism. <sup>166</sup> Three alleles (ε2, ε3, and ε4) determine three isoforms (ApoE2, ApoE3, and ApoE4) resulting in six ApoE genotypes (E2/2, E3/3, E4/4, E2/3, E2/4, E3/4). Overall homozygosity for the ε2 allele in one study was associated with dyslipidemia, but not NAFLD. <sup>167</sup> In a subgroup of nonobese individuals, the ε2 allele and the E2/3 genotype were more prevalent in controls, suggesting this allele might be protective. <sup>167</sup> Consistent with this result, the ApoE3/3 genotype was associated with NASH in a Turkish cohort, whereas ApoE3/4 was protective. <sup>168</sup>
Steatohepatitis		
Oxidative stress		
HFE	Hereditary hemochromatosis protein	Hepatic iron accumulation promotes oxidative stress. Two studies, examining 177 patients, reported carriage of an HFE polymorphism (rs1800562, encoding Cys282Tyr) that was associated with more severe steatohepatitis and advanced fibrosis. <sup>169,170</sup> However, three other studies <sup>171-173</sup> have not shown increased carriage of either the Cys282Tyr or His63Asp (rs1799945) mutations. Meta-analyses have also provided conflicting results, with the latest finding no evidence of an effect. <sup>174,175</sup>
GCLC; GCLIM	Glutamate-cysteine ligase catalytic unit; glutamate-cysteine ligase regulatory unit	Glutamate-cysteine ligase (γ-glutamyl cysteine synthetase) is the rate-limiting step in glutathione synthesis; absence of Gclc causes steatosis and liver failure in mice. <sup>176</sup> A study of 131 patients with NAFLD found the GCLC promoter region polymorphism (-129C > T, rs17883901) was associated with steatohepatitis compared with simple steatosis. <sup>160</sup>
ABCC2	ATP-binding cassette, subfamily C (CFTR/MRP), member 2	Association studies support a role for ABCC2 (also known as MRP2), which facilitates terminal excretion and detoxification of endogenous and xenobiotic organic anions, including lipid peroxidation products. <sup>177</sup>
SOD2	Superoxide dismutase [Mn], mitochondrial	Carriage of the nonsynonymous SNP (rs4880, encoding Ala16Val) has been associated with advanced hepatic fibrosis in NAFLD in both Japanese <sup>124</sup> and European <sup>125</sup> cohorts.

Table 3 (Continued)

Gene	Protein	Study details and comments
Endotoxin response		
TLR4	Toll-like receptor 4	Study of a spontaneous <i>Tlr4</i> null mutation in C3H/1 mice has established the contribution of TLR4/endotoxin to NAFLD pathogenesis in the laboratory. <sup>178</sup> <i>TLR4</i> polymorphisms (rs4986791 and rs4986790) influence hepatitis-C related fibrosis, <sup>179,180</sup> but no association with NAFLD and either <i>TLR4</i> or <i>NOD2</i> (bacterial cell wall peptidoglycan receptor) variants has been found.
CD14	Monocyte differentiation antigen CD14	CD14 is a lipopolysaccharide receptor expressed on monocytes, macrophages, and neutrophils that enhances TLR4 endotoxin signaling. An association with a promoter-region polymorphism (−159C > T, rs2569190) that increases <i>CD14</i> expression has been reported. <sup>181,182</sup>
Cytokines		
TNF	Tumour necrosis factor	A <i>TNF</i> (−238G > A, rs361525) promoter polymorphism has been associated with NASH, <sup>183,184</sup> suggesting a primary role in the transition from steatosis to steatohepatitis; a separate study, found that two other promoter region polymorphisms (−1031T > C, rs1799964 and −863C > A, rs1800630) were more common in NASH than steatosis, but were no more common in NAFLD than a control population. <sup>184</sup>
IL6	Interleukin 6	An <i>IL6</i> promoter region polymorphism (−174G > C, rs1800795) has been associated with NASH. <sup>185</sup>
IFNL4	Interferon lambda 4	The intronic rs12979860 SNP in <i>IFNL4</i> is a strong predictor of fibrosis in an etiology-independent manner, including a cohort of 488 NAFLD cases. Those with rs12979860 CC had greater hepatic inflammation and fibrosis. <sup>186</sup>
Fibrosis		
AGTR1	Type-1 angiotensin II receptor	Two studies have linked the <i>ATGR1</i> rs3772622 SNP with grade of steatohepatitis and stage of fibrosis, with the most recent study also suggesting an interaction with <i>PNPLA3</i> genotype. <sup>187,188</sup>
<i>KLF6</i>	Kruppel-like factor 6	The <i>KLF6</i> −IVS1−27G > A (rs3750861) SNP has been associated with milder NAFLD-related hepatic fibrosis in three separate European cohorts. <sup>130</sup>

Source: Modified from Anstee QM, Day CP. The genetics of NAFLD. *Nat Rev Gastroenterol Hepatol* 2013;10(11):645–655.<sup>14</sup>

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; FATP, fatty acid transport protein; HTDC, hepatic triglyceride content; LD, linkage disequilibrium; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; SNP, single nucleotide polymorphism; TG, triglyceride; T2DM, type 2 diabetes mellitus; VLDL, very low density lipoprotein.

expense of increased risk of atherosclerosis and cardiovascular disease or vice versa (► Fig. 3).<sup>117</sup> It is widely accepted that NAFLD is associated with cardiovascular disease, indeed the majority of patients with NAFLD will ultimately die a cardiovascular rather than a liver-related death.<sup>3,10,57</sup> However, these conditions can be dissociated: Individuals carrying the minor (T) allele of *TM6SF2* rs58542926 (167K) appear prone to developing NAFLD with advanced fibrosis and so are more likely to experience liver-related rather than cardiovascular morbidity and mortality.<sup>43,50,107</sup> Conversely, carriage of the C-allele is associated with dyslipidemia and cardiovascular disease.<sup>107,115</sup> As yet, it remains unclear if the effects of *TM6SF2* variants are sufficient to eclipse the many environmental and other genetic factors that determine disease outcome at an individual level, and so merit inclusion of genotype testing within a stratified medicine approach. Nevertheless, further study of this and other NAFLD-associated human genetic variants will greatly inform our understanding of the pathophysiology and interrelationship between NAFLD and its associated metabolic traits.

### Other Genetic Modifiers in NAFLD

The current understanding of disease pathogenesis has been achieved through both clinical research and the translational study of specific animal models.<sup>12</sup> It is generally accepted that the initiating events in NAFLD are dependent on the development of obesity and insulin resistance.<sup>3</sup> Together, these produce an increased free fatty acid (FFA) flux within the liver which in turn places hepatocytes under considerable metabolic load and promotes hepatocyte lipotoxicity, increased oxidative stress secondary to free radicals produced during  $\beta$ - and  $\omega$ -FFA oxidation, and endoplasmic reticulum stress. Hepatocellular TAG accumulation (steatosis) is a histologically apparent epiphenomenon reflecting these metabolic changes and is best considered an early adaptive response through which potentially lipotoxic FFAs are partitioned into relatively stable intracellular TAG stores.<sup>118</sup> Ultimately, these insults combine with the additive effects of endotoxin/TLR4 induced Kupffer cell cytokine release and immune mediated hepatocellular injury to induce cellular damage and activate cell death pathways, marking the transition to steatohepatitis.<sup>119–121</sup> If these processes persist stellate cell activation, collagen deposition and hepatic fibrosis occur.<sup>122</sup>

Potential genetic modifiers therefore fall into four broad categories: (1) those that influence glucose metabolism and insulin sensitivity; (2) those that perturb the handling of fatty acids and the accumulation of TAG in the liver; (3) those that determine progression to NASH (for example, modifiers of oxidative stress, endotoxin response or cytokine and adipokine activity); and (4) those that might influence hepatic fibrogenesis.<sup>13</sup> Drawing on the results of the GWAS discussed earlier and the numerous candidate gene studies that have been reported, ► **Table 3** summarizes the genes that have been associated with aspects of the NAFLD phenotype according to this schema. These are discussed in depth in our previous reviews of the field.<sup>13,14</sup> It should be remembered, however, that only a minority of the

genes that have been associated with NAFLD either in GWAS or through candidate-gene analysis have been independently validated and can be considered of proven importance. Indeed, if only those genes that have been robustly validated in large independent studies or through the use of transmission disequilibrium testing<sup>123</sup> are considered, the list only includes patatin-like phospholipase domain-containing 3 (*PNPLA3*),<sup>26,62–66</sup> transmembrane 6 superfamily member 2 (*TM6SF2*),<sup>43,50,107</sup> mitochondrial superoxide dismutase 2 (*SOD2*),<sup>124,125</sup> phosphatidylethanolamine N-methyltransferase (*PEMT*),<sup>126,127</sup> fatty acid desaturase 1 (*FADS1*)<sup>128</sup> and Kruppel-like factor-6 (*KLF6*).<sup>129,130</sup>

### Conclusions

Nonalcoholic fatty liver disease is a complex disease in which subtle interpatient genetic variations and environmental factors interact to determine disease phenotype and progression. The relative importance of these factors will vary between populations depending on background modifier genes, lifestyle choices/challenges, and other factors such as the intestinal microbiome. Beyond identifying associations, it is important to understand the mechanisms through which these variations exert effects and to translate these findings into clinical utility where possible.

### Abbreviations

<sup>1</sup> H-MRS	proton magnetic resonance spectroscopy
ALT	alanine transaminase
AST	aspartate aminotransferase
<i>ATGL</i>	adipose triglyceride lipase
BMI	body mass index
<i>FDFT1</i>	farnesyl diphosphate farnesyl transferase 1
GCKR	glucokinase regulator
GWASs	genome-wide association studies
HCC	hepatocellular carcinoma
HTGC	hepatic triglyceride content
LYPLAL	lysophospholipase-like
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
<i>PNPLA3</i>	patatin-like phospholipase domain containing 3
SNPs	single nucleotide polymorphisms
T2DM	type 2 diabetes mellitus
TAG	triacylglycerol
TG	triglyceride
<i>TM6SF2</i>	transmembrane 6 superfamily member 2
VLDL	very low-density lipoprotein

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