TM6SF2: A Novel Genetic Player in Nonalcoholic Fatty Liver and Cardiovascular Disease

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Transmembrane 6 superfamily member 2 (*TM6SF2*) is located on chromosome 19 (19p12) and encodes for a protein of undetermined function. Genetic studies have reported the association between a nonsynonymous variant in *TM6SF2* (E167K, rs58542926) with hepatic triglyceride content and its impact on the cardiovascular system. Clinical and epidemiological studies have confirmed the role of *TM6SF2* in the development of nonalcoholic fatty liver disease (NAFLD). Recently, *TM6SF2* was also shown to play an important role in promoting hepatic fibrosis and hepatocellular cancer in mouse models. This review aims to capture the physiological role of *TM6SF2* in the regulation of lipid metabolism and its involvement in cardiometabolic diseases. (*Hepatology Communications* 2022;6:448-460).

Nonalcoholic fatty liver disease (NAFLD) is a form of chronic liver disease characterized by abnormal accumulation of triglycerides (TGs) in the liver.[1,2] Currently, NAFLD is the second most common etiology of liver transplantation in the United States and is projected to be the leading cause by the end of this decade.[3] The earliest stage of NAFLD is hepatic steatosis, which is often self-limiting and benign; this can progress to chronic inflammation, known as nonalcoholic steatohepatitis (NASH), cirrhosis, and eventually hepatocellular carcinoma (HCC), in a fraction of patients.[2,4] Epidemiological studies have shown that a significant portion of patients with NAFLD (~20%) progress to NASH,[5] but the risk determinants of disease progression remain unclear. Obesity and type 2 diabetes mellitus are the two major risk factors in the development of NAFLD.[2] Disease susceptibility also varies among individuals and different ethnicities, underlying a genetic basis for the disease.

Evidence from twin studies document the role of heritability in hepatic fat accumulation and presence of NAFLD.[6,7] A seminal study conducted on a multi-ethnic cohort in the Dallas Heart Study (DHS) further confirmed the genetic basis for the development of NAFLD.[8] In a first, a study showed variation of the degree of accumulation of TGs in the liver across races. Genome-wide association study (GWAS) on the same cohort identified patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) and the I148M variant (rs738409) as the predominant genetic risk factor associated with development of

Abbreviations: ABCG, adenosine triphosphate-binding cassette subfamily G member; ALT, alanine aminotransferase; APOB, apolipoprotein B; ASCVD, atherosclerotic cardiovascular disease; CVD, cardiovascular disease; CVS, cardiovascular system; DGAT, diacylglycerol O-acyltransferase; DHS, Dallas Heart Study; EBP, emopamil binding protein; ER, endoplasmic reticulum; ERGIC, endoplasmic reticulum–Golgi intermediate compartment; GWAS, genome-wide association study; HCC, hepatocellular carcinoma; HDL-C, high-density lipoprotein cholesterol; KO, knockout; LDL-C, low-density lipoprotein cholesterol; mRNA, messenger RNA; MTTP, microsomaltriglyceride transfer protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PCs, phosphocholine; PUFA, polyunsaturated fatty acid; TC, total cholesterol; TG, triglyceride; TM6SF2, transmembrane 6 superfamily member 2; VLDL, very low-density lipoprotein.

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NAFLD.[9,10] A point mutation (rs58542926, c.499 C>T, P. Glu167Lys, E167K) in another gene, transmembrane 6 superfamily 2 (*TM6SF2*), was also independently associated with elevated liver TGs, higher circulating levels of alanine aminotransferase (ALT), and lower levels of low-density lipoprotein cholesterol (LDL-C).[11-13] The association between the *TM6SF2* E167K variant and NAFLD was also confirmed in subsequent studies.[14-16] The minor (T) allele is more frequent in East Asians (approximately 34%) and Europeans (approximately 26%) and less common in Hispanics/Latinos (approximately 10%) and Africans (6%, Genome Aggregation Database).[17] Existing literature also reports a greater proportion of patients who are lean with NAFLD carrying the *TM6SF2* rs58542926 (T) allele than patients who are obese/overweight with NAFLD,[18] further underscoring its independent role in the development of the disease.

The deleterious effect of the *TM6SF2* E167K mutation in NAFLD is paradoxical in the context of the cardiovascular system (CVS), where it has been associated with a lower risk of cardiovascular disease (CVD).[12,14-16] Mendelian randomization studies have also confirmed the cardioprotective effect of the *TM6SF2* E167K variant.[19] Burgeoning evidence has established an independent role of NAFLD in the risk of developing CVD,[20-22] although mechanistic understanding remains obscure. The cardioprotective role of the *TM6SF2* E167K variant implies a different disease pathogenesis with this genetic variant.[23] Proper understanding of the physiological role of *TM6SF2* in the body will aid us in providing insights to resolve the conundrum.

Characterization of *TM6SF2*

GENE AND PROTEIN

TM6SF2, first reported in 2000,[24] is located on chromosome 19p12 and encodes a protein containing 377 or 350 amino acids (two isoforms produced by alternative splicing). The protein is predicted to have seven to 10 transmembrane domains,[11] while an additional expanded emopamil binding protein (EBP) superfamily (EXPERA) domain was found to be conserved, along with 3-β-hydroxysteroid-8,7-iso merase, through computational analysis.[25] This can potentially indicate its role as an enzyme in cholesterol metabolism. The gene is moderately conserved across species, with the human protein showing 99.73%, 78.4%, and 78.57% homology with chimpanzee, mouse, and rat, respectively (https://www.ncbi.nlm.nih.gov/homologene/?term=Homo+sapiens+TM6SF2). A nonsynonymous point mutation in *TM6SF2* (rs58542926, c.499 C>T) causes a glutamine to lysine substitution at residue 167 (p. Glu167Lys, E167K), resulting in a misfolded protein, accelerated protein degradation, and reduced protein levels in the body.[11]

TISSUE DISTRIBUTION AND REGULATION

TM6SF2 messenger RNA (mRNA) is abundantly expressed in liver, intestine, and kidney in both human and mouse.[11,13,26] However, TM6SF2 protein in mouse is around 10-fold higher in the small intestine

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than liver.[26] Expression of full-length TM6SF2 protein containing a C-terminal green fluorescent protein tag in human hepatoma (Huh7) cells revealed perinuclear lattice-like staining and considerable overlap with protein disulfide isomerase (endoplasmic reticulum [ER] marker) and ER-Golgi intermediate compartment (ERGIC)-53 (ERGIC marker).[13] However, limited overlap was observed between TM6SF2 and Golgi marker Golgin subfamily B member 1 (GIANTIN),[13] which led us to hypothesize that the subcellular localization of TM6SF2 is limited to the ER and ERGIC. However, subcellular fractionation of murine liver and biochemical analysis showed the presence of TM6SF2 in the ER and Golgi.[26] The results were further validated in primary murine hepatocytes through confocal microscopy, where TM6SF2 colocalized with an ER marker (calnexin) and Golgi markers (receptor-binding cancer antigen expressed on SiSo cells [RCAS1] and Giantin) but not with boron-dipyrromethene staining.[26] Given the technical challenges associated with the isolaton of ER and Golgi from mouse liver, contamination of ER with the Golgi compartment could not be completely ruled out. Unlike what was observed previously by immunocytochemistry, we recently found TM6SF2 mainly localized to the smooth ER but not to Golgi.[27] In view of these conflicting lines of evidence, further research is required to confirm the subcellular localization of TM6SF2. Lei and colleagues[28] reported carbohydrate-responsive element-binding protein (*ChREBP*)-mediated transcriptional and translational up-regulation of TM6SF2 protein.[28] However, no change in levels of TM6SF2 protein was observed after fasting and refeeding the mice with a normal chow diet.[26] An explanation for this discrepancy can be due to up-regulation of hepatic *ChREBP* in response to high-sucrose diet refeeding following fasting.[29] Further experiments are required to validate the nutritional regulation of *Tm6sf2*.

Clinical Relevance of *TM6SF2*

RELATIONSHIP BETWEEN *TM6SF2* E167K VARIANT AND NAFLD

Genetic evidence from multiple GWASs demonstrates the association of single nucleotide polymorphism (rs10401969) in the 19p12 locus with plasma levels of TG and total cholesterol (TC).[30-35] In addition, GWASs established the association between the 19p12 locus and NALFD,[36,37] coronary heart disease,[34,38] and diabetes.[39,40] However, no individual gene at this locus had been reported to regulate plasma lipids at that time. Mahdessian et al.[13] performed gene expression analysis on 206 human liver tissues and reported the highest expression of TM6SF2 mRNA among genes in the 19p12 region. Subsequently, an expression quantitative trait locus analysis of rs10401969 was mapped to the expression of TM6SF2 mRNA (P = 0.0018), suggesting that *TM6SF2* may play a dominant functional role in the NAFLD phenotype.

A growing body of evidence from population genetic studies implicates the *TM6SF2* E167K variant as an independent risk factor for NAFLD (Table 1). A seminal study involving three independent populations (DHS, Dallas BioBank, and Copenhagen City Heart Study), confirmed the association between the *TM6SF2* E167K variant and increased hepatic TG content and ALT.[11] Subsequent studies in larger multi-ethnic cohorts, except one,[41] validated the association of the *TM6SF2* E167K variant with NAFLD.[15,41-45] A possible explanation of the null association between the *TM6FS2* E167K variant and NAFLD in Hispanics could be due to small sample size and lack of statistical power. However, the relationship between the *TM6SF2* E167K variant and NAFLD severity, fibrosis, cirrhosis, and HCC is less consistent.

An initial study by Liu et al.[46] demonstrated an association between the *TM6SF2* E167K variant with hepatic steatosis and histologic stage of fibrosis but no correlation with histopathological evidence of steatohepatitis and HCC. Similar results were reported by other groups, linking the *TM6SF2* E167K variant with hepatic steatosis but not with fibrosis or histopathological steatohepatitis.[14,47-50]

However, in a recent large cross-sectional study in a European cohort consisting of 1,201 patients, Dongiovanni and colleagues[16] showed a positive association between the *TM6SF2* E167K variant and hepatic steatosis, steatohepatitis, and fibrosis. A meta-analysis of four studies and 4,325 patients also confirmed the increased risk of steatosis, fibrosis, and cirrhosis in carriers of the variant.[51]

The conflicting nature of the evidence can be attributed to the cumulative risk due to the presence of confounding risk factors, like alcoholic liver disease

TABLE 1. CLINICAL RELEVANCE OF TM6SF2

Reference	Sample Size	Ethnicity/ Place of Study	Plasma Lipids	Liver Enzymes	HTGC/Method	NASH	Fibrosis	Cirrhosis	HCC	CVS
Kozlitina et al.[11]	DHS (n = 2,736)	non-Hispanic African Americans, non-Hispanic, European Americans, Hispanics and other ancestry	LDL-C↓ and TG↓	ALT↑	↑/^{1}H-MRS	NM	NM	NM	NM	NM
	Dallas BioBank (n = 8,585)	European-Americans	LDL-C↓ and TG↓	ALT↑	↑/^{1}H-MRS	NM	NM	NM	NM	NM
	Copenhagen City Heart Study and Copenhagen General Population Study (n = 73,532)	Denmark	LDL-C↓ and TG↓	ALT↑ and AST↑	↑/^{1}H-MRS	NM	NM	NM	NM	NM
Holmen et al.[12]	N = 92,605	Europeans	TC↓ and TG↓	NM	NM	NM	NM	NM	NM	MI↓
Liu et al.[46]	Discovery cohort (n = 349)	European Caucasian	NM	NM	↑/biopsy	↑	↑	Not clear	NM	NM
	Validation cohort (n = 725)	European Caucasian	NM	NM	↑/biopsy	NA	↑	Not clear	NM	NM
	NAFLD-HCC cohort (n = 99)	European Caucasian	NM	NM	NM	NM	NM	NM	↑	NM
Wong et al.[47]	HK-MRS (n = 922)	Chinese	TC↓, LDL-C↓ and TG↓	NM	↑/^{1}H-MRS	NM	NA	NA	NM	NM
Sookoian et al.[14]	N = 361	Argentina	TC↓, LDL-C: NA, and TG: NA	NA	↑/biopsy	NM	NA	NM	NM	CVD risk↓
Dongiovanni et al.[16]	Liver biopsy cohort (n = 1,201)	Italy, Finland	TC↓, LDL-C: NA and TG↓	NA	↑/biopsy	↑	↑	NM	NM	NM
	carotid atherosclerosis cohort (427)	Italy	NM	NM	NM	NM	NM	NM	NM	Carotid plaques↓
	SOS cohort (n = 1,819)	Swedish	ALT↑ and AST↑	NM	NM	NM	NM	NM	NM	CVD events↓
Zhou et al.[45]	N = 300	Finnish	LDL-C and TG: NA	ALT and AST: NA	↑/^{1}H-MRS	NM	NM	NM	NM	NM
Akuta et al.[48]	Patients with biopsy-proven NAFLD (n = 211)	Japanese	NM	NM	NM	NA	NA	NM	NM	NM
Goffredo et al.[41]	Children/adolescents with obesity (n = 402)	Caucasians	TC↓, and LDL-C↓	↑ trend	↑/MRI	NM	NM	NM	NM	NM
	Children/adolescents with obesity (n = 266)	African Americans	NA	NA	↑/MRI	NM	NM	NM	NM	NM
	Children/adolescents with obesity (n = 289)	Hispanics	TC↓, and LDL-C↓	ALT↑ and AST↑	↑/MRI	NM	NM	NM	NM	NM

TABLE 1. *Continued*

Reference	Sample Size	Ethnicity/ Place of Study	Plasma Lipids	Liver Enzymes	HTGC/Method	NASH	Fibrosis	Cirrhosis	HCC	CVS
Grandone et al.[57]	Children/adolescents with obesity (n = 1,010)	Italy	TC↓, LDL-C↓ and TG↓	ALT↑ and AST: NA	↑/ultrasound	NM	NM	NM	NM	NM
Mancina et al.[56]	Children/adolescents with obesity (n = 423)	Italy	NM	NA	↑/ultrasound	NM	NM	NM	NM	NM
O'Hare et al.[60]	GHS bariatric surgery cohort (n = 983)		NA	NM	↑/biopsy	↑	↑	NA	NM	NM
	ACDRP cohorts (n = 3,556)	Caucasian	TC↓, LDL-C↓ and TG↓	NA	NM	NM	NM	NM	NM	NM
Krawczyk et al.[49]	NAFLD Clinical Study Group project (n = 515)	German	NM	ALT↑ and AST↑	↑/MRI	NM	NA	NM	NM	NM
Liu et al.[15]	N > 300,000	European, African, South Asian, Hispanic or other ancestry	TC↓ and TG↓	NM	↑/CT	NM	NM	NM	NM	CAD↓
Basyte-Bacevice et al.[50]	N = 1,012	Lithuania	NM	NM	NM	NM	NA	NA	NM	NM
Anstee et al.[42]	Histologically characterized cohort (1,483 biopsied NAFLD cases and 17,781 controls)	Europeans	NM	NM	↑/biopsy	↑	NM	NM	NM	NM
Parisinos et al.[43]	UK BioBank Cohort (n = 14,440)	Europeans	LDL-C↓ and TG↓	ALT↑ and AST↑	↑/MRI	NM	NM	↑	NM	CAD↓
Chen et al.[44]	UK BioBank (1,088 cases vs. 407,873 controls) and Michigan Genomics Initiative (875 cases of cirrhosis vs. 30,346 controls)	UK, US	LDL-C↓ and TG↓	ALT↑	NM	NM	NM	↑	NM	NM

Abbreviations: ↑, increase or positive association; ↓, decrease or negative association; ACDRP, Amish Complex Disease Research Program; AST, aspartate transaminase; CAD, coronary artery disease; CT, computed tomography; GHS, Geisinger Health System; HK-MRS, Hong Kong, magnetic resonance spectroscopy; ^{1}H-MRS, proton nuclear magnetic resonance spectroscopy; HTGC, hepatic triglyceride content; MI, myocardial infarction; MRI, magnetic resonance imaging; NA, no association; NM, not mentioned; SOS, Obese Subjects Study; UK, United Kingdom; US, United States.

and hepatitis C infection. The lower allele frequency and relatively small size of cohorts could explain conflicting results. A GWAS identified the *TM6SF2* E167K variant as a risk locus for alcohol-related cirrhosis in individuals of European descent, with subsequent validation in two independent European cohorts.[52] Moreover, the same variant was strongly associated with alcohol-related and non-hepatitis B HCC.[53,54] Future prospective studies with proper phenotyping for NAFLD and genotyping, apart from comparison between groups that are well matched for risk factors for chronic liver disease, need to be conducted to ascertain the risk of disease severity of NASH and of fibrosis with the *TM6SF2* variant. Finally, a Mendelian randomization study using genetic risk variants for NAFLD also confirmed a robust causal relationship between hepatic fat content and fibrosis, which further points toward the role of *TM6SF2* in accelerating liver fibrosis.[55]

Apart from adults, the *TM6SF2* E167K variant has also been associated with NAFLD in children and adolescents.[56,57] Using imaging modalities (ultrasonography and magnetic resonance imaging) and liver biopsies, independent groups have demonstrated the association between the *TM6SF2* E167K variant and liver fat content[11,16,45,46] and severity of liver disease.[14,41,56]

TM6SF2 E167K VARIANT AND CVD

The American College of Cardiology/American Heart Association Atherosclerotic Cardiovascular Disease (ASCVD) Risk Estimator identifies dyslipidemia, including elevated plasma TC, LDL-C, and TG levels and decreased high-density lipoprotein cholesterol (HDL-C) levels, as independent risk factors for CVD.[58] In a seminal GWAS that included 5,643 Norwegians, a strong association between the *TM6SF2* E167K variant and reduction of serum TC and TG and concomitant reduced risk of myocardial infarction was described (Table 1).[12] The results have since been replicated in subsequent studies involving independent cohorts, highlighting the paradoxical effect of the *TM6SF2* variant in heart and liver.[11,15,16] The cardioprotective effect of the *TM6SF2* variant is primarily due to its effect on a more favorable plasma lipid profile.[16] A meta-analysis that included 101,326 subjects showed that carriers of the KK genotype of the *TM6SF2* E167K variant had lower levels of TC and LDL-C than those homozygous for the *TM6SF2* EE genotype.[59] This has been supported by data from the Amish Complex Disease Research Program cohort, which reported an increase in plasma level of HDL-C in addition to a decrease in TC, LDL-C, and TG in patients with the *TM6SF2* E167K variant.[60] The relation between dyslipidemia and subclinical atherosclerosis has been well documented.[61] Carotid plaque measurements, which are a surrogate marker of subclinical atherosclerosis, were used by Dongiovanni and colleagues[16] in patients with NASH. While the overall risk of cardiovascular events increased in patients with NASH, *TM6SF2*-mediated NASH was noted to have lower subclinical atherosclerosis as well as a lower incidence of cardiovascular events. However, the cardioprotective effect of the *TM6SF2* E167K variant was lost after adjusting for plasma TC levels,[16] implying a *TM6SF2*-induced favorable plasma lipid profile as the major contributor. Inflammation plays a significant role in subclinical ASCVD.[62,63] C-reactive protein, a well-validated biomarker of subclinical ASCVD, was markedly reduced in patients with NAFLD carrying the *TM6SF2* E167K variant when compared to controls.[14] The effect of the *TM6SF2* NAFLD risk variant over CVS requires more attention from both clinicians and researchers. Aggressive management of hepatic steatosis in patients with NAFLD may negate its positive effect on the heart. Replication of the cardioprotective effects of *TM6SF2* in longitudinal studies and better mechanistic understanding of its role in hepatic steatosis will aid in therapeutic manipulation of downstream effects of *TM6SF2* without compromising its beneficial effect on the CVS.

Preclinical Studies Involving *TM6SF2*

MURINE MODELS

To explore the mechanistic role of *TM6SF2* in NAFLD pathogenesis and blood lipid regulation, murine models of *Tm6sf2* knockdown or knockout (KO) were established. Kozlitina et al.[11] knocked down TM6SF2 by injecting adenovirus-associated virus (AAV) into the tail vein of C57BL/6J mice to achieve significant reduction of protein translation.

Knockdown of TM6SF2 resulted in significant lowering of plasma TC and TG levels in the knockdown group compared to control, while their liver TG levels increased significantly. The difference in liver TG deposition was more conspicuous when fed with a high-sucrose diet.[11] Although ALT levels (marker of hepatic dysfunction) were significantly increased in *Tm6sf2* KO mice, there was no evidence of inflammation, differential expression of fibrosis-associated genes, and histopathological characteristics of liver injury.[26] Another group used adenovirus with short hairpin RNAs to target the *Tm6sf2* coding region in C57BL/6J mice; they reported a significant decrease in circulating TC levels with no concurrent changes in liver TG and hepatic functions.[12] A possible explanation of the discrepancy in results could be due to either a different mode of interfering RNA delivery, extent of the mRNA knockdown, or variable hepatic TM6SF2 protein levels. To circumvent these limitations, permanent gene deletion of established *Tm6sf2* employing the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system was undertaken to create *Tm6sf2* KO mice.[64] Under a normal diet, serum TC and HDL-C were significantly reduced compared to their wild-type littermates, with no cholesterol or TG deposition in the liver,[64] which is inconsistent with previous reports.[26] We found inactivation of TM6SF2 resulted in hepatic steatosis in rat under a normal diet.[27] More recently, two other independent groups reported that whole-body or hepatic ablation of *Tm6sf2* in mice leads to increase hepatic TG accumulation.[65,66] Liver-specific inactivation of TM6SF2 was reported to promote hepatic steatosis and fibrosis and accelerate development of HCC.[66] Whether TM6SF2 plays a role in development of hepatic fibrosis and HCC needs further investigation.

Ehrhardt et al.[67] achieved transient genetic overexpression of *Tm6sf2* in mice by injecting AAV into wild-type C57BL/6J mice; they noted significant reduction of plasma TG, TC, and HDL. Moreover, *Tm6sf2* overexpression resulted in accumulation of TG in the liver but not cholesterol.[67] However, stable hepatic overexpression of human *TM6SF2* in mice resulted in increased circulating plasma lipids (TC and HDL-C) and hepatic TG and cholesterol.[64] Of note, both the KO and overexpression of *Tm6sf2* in murine models result in hepatic steatosis, implying a more complex association between mutant genotype and phenotype, which could not be explained through merely gain or loss of function alone. Because TM6SF2 is localized in ER and binding to apolipoprotein B (ApoB), there is a high possibility that overexpression of *Tm6sf2* may sequester ApoB-containing lipoproteins in ER. Ehrhardt et al.[67] showed *Tm6sf2* overexpression reduced ApoB secretion and resulted in its accumulation within ER, which is consistent with this hypothesis.

Data regarding the cardiovascular effects of murine models of *Tm6sf2* KO remain lacking, which necessitates further research.

ZEBRAFISH MODEL

The expression of *tm6sf2* is confined to liver and intestine in zebrafish. Using the CRISPR/Cas9 system to inactivate *tm6sf2* expression in zebrafish, O'Hare et al.[60] reported significant TG accumulation in the liver and intestine.

Mechanism of Action of *TM6SF2* in Lipid Metabolism

TG IN *TM6SF2* AND HEPATIC LIPID METABOLISM

Liver and intestine are the two major sources of lipids in the body. The intestine secretes lipid in the form of chylomicrons, while liver can synthesize and package lipids into very low-density lipoprotein (VLDL) and secrete them into circulation.[68,69] *TM6SF2* is predominantly expressed in the small intestine and liver, raising the possibility that *TM6SF2* may regulate lipid metabolism in these organs. Liver lipids are mainly derived from *de novo* synthesis of fatty acids or absorption of remnant lipoprotein particles and free fatty acids from the circulation. A fraction of hepatic lipids is mainly used for hepatic energy production, and another part is secreted into circulation in the form of VLDLs. Hepatic steatosis due to loss of *TM6SF2* can be either due to abnormality in TG synthesis or secretion of VLDL-TG or a combination of both. Inactivation of *Tm6sf2* in mice did not affect sterol responsive element binding protein (SREBP-1 and SREBP-2), the master transcriptional regulator

of fatty acid and cholesterol biosynthesis,[26] ruling out the possibility of excess synthesis causing hepatic steatosis in *Tm6sf2* KO mice. Increased hepatic fat and decreased circulating lipids in *Tm6sf2* KO mice are consistent with a defect in VLDL-TG secretion, conferred by loss of functional protein. The defect of VLDL-TG secretion can be explained through the following two potential mechanisms: first, reduction in the number of secreted VLDL particles from the liver as in the microsomal triglyceride transfer protein (*MTTP*) KO model[70,71]; and second, through the reduction in the TG content of VLDL particles while the number of VLDL particles remains constant.[72] APOB concentration is a surrogate marker of the number of VLDL particles, and TG content is a reflection of the particle size. Smagris et al.[26] found that VLDL particles of *Tm6sf2* KO mice were smaller in size than wild-type mice and secretion of VLDL-TG was significantly reduced without any reduction in plasma APOB levels, indicating that defective VLDL-TG but not APOB secretion may be predominantly involved in eliciting the phenotype. The role of *TM6SF2* on APOB secretion was also explored *in vitro*. *TM6SF2* knockdown in human cell lines (Huh7 and HepG2) results in a remarkable decrease in TG secretion with a modest reduction in ApoB levels.[13] However, in a pulse-chase experiment to monitor APOB secretion, the investigators observed that the level of newly synthesized and secreted APOB100 in plasma of *Tm6sf2* KO mice was similar to that of the wild type while a mild increase in APOB48 was observed in KO mice,[26] which was also validated in primary hepatocytes.[66] No changes in hepatic APOB levels were reported in *Tm6sf2* KO mice.[26] These findings indicate that loss of *TM6SF2* function results in decreased VLDL lipidation without alteration of APOB synthesis or secretion.

Existing data suggest that lipidation of VLDL is a two-step process.[73] The first step involves lipidation associated with APOB synthesis taking place in the rough ER, which requires the presence of the MTTP protein.[71] The second step in the lipidation process involves further lipidation of nascent VLDL. In the event of any defect in the first lipidation step, the nonlipidated APOB protein will get degraded and will lead to a significant reduction in plasma APOB level.[71] Data from transmission electron microscopy of liver showed a decrease in the size of the nascent VLDL particles in Golgi of liver-specific *Tm6sf2* KO mice and undetectable levels of VLDL particles in the ER or Golgi of liver-specific *Mttp* KO mice, indicating *TM6SF2* may be involved in the second step of lipidation. *In vitro* data from *TM6SF2* knockdown in HuH7 and HCC (HepG2) cells showed a mild reduction in secretion of APOB.[13] However, as mentioned above, APOB levels in liver and serum of *Tm6sf2* KO mice did not show any decrease in comparison to wild-type mice,[26,66] suggesting that *Tm6sf2* may not be involved in the early lipidation of VLDL. Reduced APOB secretion was also reported in carriers of the *TM6SF2* E167K genetic variant in human hepatic three-dimesional spheroids.[74] Possible explanations for the difference in APOB levels could be functional change in cellular metabolism and interspecies difference in the presence of APOB subtypes between humans and mouse. More recently, it was reported that TM6SF2 interacts and stabilizes APOB.[65] Knockout of *Tm6sf2* leads to decreased hepatic APOB protein level in mice according to the time of day the mice were fasted.[65] However, under both conditions, plasma TC and TG were increased in the liver and decreased in serum of *Tm6sf2*-deficient mice,[65] ruling out any dependence of hepatic steatosis phenotype in *Tm6sf2* KO mice on "time-of-day" fasting.

Phospholipids and polyunsaturated fatty acids (PUFAs) are critical players in the second stage of lipidation of VLDL.[72] Luukkonen and colleagues[75] reported reduction in the level of PUFAs in liver and serum TGs and liver phosphocholine (PCs) in patients carrying the *TM6SF2* E167K variant, but hepatic free fatty acids were relatively enriched in PUFA. Concomitantly, decreased incorporation of PUFAs into TGs and PCs in *TM6SF2* knockdown hepatocytes was reported.[76] Another study also reported a significant reduction of polyunsaturated lipid species and an increase in saturated and monounsaturated species in HuH7 cells following *TM6SF2* knockdown.[76] They reported relative and absolute arachidonic acid (AA, 20:4n-6) depletion in the PCs.[76] These findings have not been confirmed in animal studies. Although marginal differences of hepatic and plasma fatty acids were observed in wild-type and *Tm6sf2* KO mice, the AA content of the PC fraction was similar between the two groups.[26]

Knockdown of *TM6SF2* in Huh7 and HepG2 cell lines also reduces transcription of diacylglycerol O-acyltransferase 1 (DGAT1) and DGAT2,[13]

which are key enzymes in TG synthesis.[77] However, there is no evidence to confirm that transcriptional perturbation of *DGAT1* and *DGAT2* leads to a decrease in translational efficiency. Taken together, the effect of *Tm6sf2* KO appears to be mediated through decreased lipidation of VLDL (Fig. 1).

CHOLESTEROL IN *TM6SF2* AND HEPATIC LIPID METABOLISM

The role of *Tm6sf2* in cholesterol metabolism has also been explored. The 3-β-hydroxysteroid-8,7-isom erase coded by *EBP* is a key enzyme in the cholesterol biosynthetic pathway.[78] TM6SF2 protein was identified as an EBP homologue and shares a functional domain with EBP.[25] In HuH7 cells, overexpression of *TM6SF2* increases cholesterol biosynthesis, while inhibition of downstream enzyme Δ7-dehydrocholesterol reductase (an enzyme converting 7-dehydrocholesterol to cholesterol at the final step of cholesterol biosynthesis) abolishes this effect.[64] Compared with wild-type mice, the expression of cholesterol metabolism-related genes (such as LDL receptor; scavenger receptor, class B type 1 [*SR-BI*]; and proprotein convertase subtilisin/kexin type 9 serine protease [*PCSK9*]) in *Tm6sf2* KO mice did not show any difference[26] except for 7-dehydrocholesterol reductase, which had a higher expression in KO mice.[64] Adenosine triphosphate-binding cassette subfamily G member (ABCG) 5 and ABCG8 can promote the removal of liver cholesterol through hepatobiliary secretion.[79] The levels of ABCG5 and ABCG8 were elevated in the livers of *Tm6sf2* KO mice in comparison to their wild-type littermates.[64] However, the underlying mechanism is unclear. These findings indicate that *TM6SF2* may also promote liver cholesterol biosynthesis.

TM6SF2 AND INTESTINAL LIPID ABSORPTION

O'Hare et al.[60] reported that carriers of the *TM6SF2* 167K allele had significantly lower postprandial serum TG level than the carriers of *TM6SF2* 167E allele after high-fat stimulation in the Amish Complex Disease Research Program. This indicated that the E167K variant is associated with lipid absorption, which was further confirmed in another independent study.[80] Smagris et al.[26] reported that *Tm6sf2* KO mice fed a high-fat diet for 12 weeks had a large accumulation of neutral lipids in jejunum but no accumulation was observed in wild-type mice. They further conducted a lipid tolerance test by providing mice corn oil (10 μL/g) through oral gavage and then measured plasma TG levels. The results showed that absorption of lipids in wild-type mice and *Tm6sf2* KO mice was similar, although the absorption peak of *Tm6sf2* KO mice was shifted compared to that of wild-type mice, suggesting impairment in the rate of intestinal lipid absorption.[26] Consequently, *TM6SF2* deficiency might be responsible for the reduced secretion of chylomicrons from intestinal cells. Furthermore, inactivation of *tm6sf2* in zebrafish can induce TG accumulation in cytoplasmic lipid droplets of intestinal epithelial cells.[60] Because the absorption of dietary lipid involves multiple steps (starting from storage of absorbed lipids to assembly of chylomicrons), the precise role of *TM6SF2* activity in the process cannot be delineated.[60]

Challenges and Future Directions

NAFLD, a burgeoning etiology of chronic liver disease in the developed world, is a complex metabolic disease leading to systemic manifestations that can also affect the cardiovascular system. Currently, therapies targeting disease progression in NAFLD are lacking. Leveraging human genetics to identify risk determinants in NAFLD has unearthed a strong association between the *TM6SF2* E167K variant and hepatic steatosis. Genetic risk variants, like *TM6SF2* E167K, have preventive and therapeutic importance in the context of NAFLD. The insidious nature of NAFLD necessitates early detection and intervention to prevent late sequelae of the disease. Genetic risk scores based on genetic variants alone or taken together may allow clinicians to screen for hepatic steatosis to identify at-risk individuals and intervene through modifiable risk factors, like dietary restriction and weight loss.[81] Incorporating genetic risk variants, such as *TM6SF2*, may aid in improving noninvasive diagnostic accuracy of NAFLD as well.[55] As shown by Paternostro et al.,[82] adding TM6SF2 to risk stratification models improved the diagnostic accuracy of the model for the prediction of advanced fibrosis. From a therapeutic standpoint, patients carrying the *TM6SF2* E167K

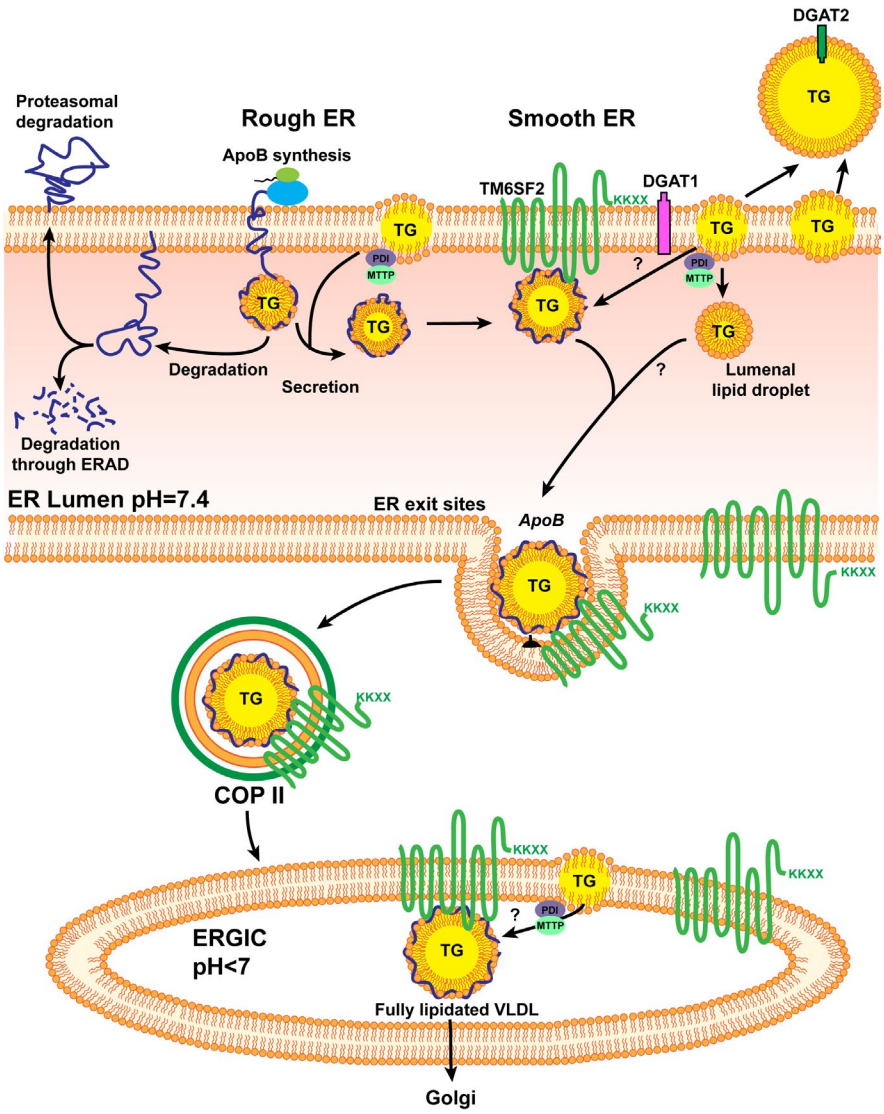

FIG. 1. The potential mechanisms of *TM6SF2* in hepatic lipid metabolism. *TM6SF2* localized to ER and ERGIC. (Adapted from Mahdessian et al., Proc Natl Acad Sci U S A 2014;111:8913-8918.). TM6SF2 interacts with APOB and is involved in the second lipidation of VLDLs. Abbreviations: COP II, coat protein complex II; ERAD, endoplasmic-reticulum-associated protein degradation.

variant can be targeted for early intervention, despite not having histologic evidence of NASH at a time point. Secondly, this genetic risk variant can be beneficial in enrolling these at-risk patients in clinical trials for the development of novel therapies for NAFLD/NASH, thereby increasing the power of these studies and addressing drug efficacy against genetic risk-mediated NAFLD.[83] Finally, therapies targeting the relevant biological pathways affected by genetic risk variants, like *TM6SF2*, may aid in reducing the increased risk of developing NAFLD in this group of patients. Primary prevention strategies, including close clinical monitoring of individuals carrying this variant, may be beneficial in reducing the disease burden in the population. An evolving body of clinical and preclinical literature implicates the role of this risk variant in reducing VLDL-TG secretion from the liver, resulting in accumulation of hepatic TGs leading to NAFLD. However, mechanistic insights about the exact role of this variant in causing NAFLD remains unknown and represents an area of active interest and research. Better understanding of the disease pathogenesis due to this risk variant may aid in developing targeted therapies to treat NAFLD. Of note, carriers

of the *TM6SF2* variant also have favorable cardiovascular outcomes. Hence, identification of downstream pathways altered by the *TM6SF2* E167K variant may aid in the disentanglement of its deleterious role in NAFLD development. It will also help in developing therapeutic strategies to retain its cardioprotective effect without offsetting the benefit of targeting the variant itself for the treatment of NAFLD.

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