TM6SF2 Gene Variant Disentangles Nonalcoholic Steatohepatitis from Cardiovascular Disease

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FOOTNOTE PAGE

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Abbreviations: NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; PNPLA3: Patatin-like phospholipase domain-containing-3; TM6SF2: Transmembrane 6 superfamily member 2; VLDL: very low density lipoproteins; SOS: Swedish Obese Subjects study

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CONFLICT OF INTEREST

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ABSTRACT

Excess hepatic storage of triglycerides is considered a benign condition, but nonalcoholic steatohepatitis may progress to fibrosis and may promote atherosclerosis. Carriers of the *TM6SF2* E167K variant have fatty liver due to reduced secretion of very low density lipoproteins. As a result, they have lower circulating lipids and reduced risk of myocardial infarction. In this study, we aimed to assess whether *TM6SF2* E167K affects liver damage and cardiovascular outcomes in subjects at risk of nonalcoholic steatohepatitis. Liver damage was evaluated in 1201 patients who underwent liver biopsy for suspected nonalcoholic steatohepatitis; 427 were evaluated for carotid atherosclerosis. Cardiovascular outcomes were assessed in 1819 controls from the Swedish Obese Subjects cohort. Presence of the inherited *TM6SF2* E167K variant was determined by Taqman assays. In the liver biopsy cohort, 188 subjects (13%) were carriers of the E167K variant. They had lower serum lipid levels than noncarriers ($P<0.05$), had more severe steatosis, necroinflammation, ballooning, and fibrosis ($P<0.05$), and were more likely to have nonalcoholic steatohepatitis (OR 1.84, 95% CI 1.23-2.79) and advanced fibrosis (OR 2.08, 95% CI 1.20-3.55), after adjustment for age, sex, body mass index, fasting hyperglycemia, and the I148M *PNPLA3* risk variant. However, E167K carriers had lower risk of developing carotid plaques (OR 0.49, 95% CI 0.25-0.94). In the Swedish Obese Subjects cohort, E167K carriers had higher ALT and lower lipid levels ($P<0.05$), and a lower incidence of cardiovascular events (HR 0.61, 95% CI 0.39-0.95). ***Conclusions:*** Carriers of the *TM6SF2* E167K variant are more susceptible to progressive nonalcoholic steatohepatitis, but are protected against cardiovascular disease. Our findings suggest that reduced ability to export very low density lipoproteins is deleterious for the liver.

With the rise in obesity rates, nonalcoholic fatty liver disease (NAFLD), the hepatic manifestation of metabolic syndrome (1), is becoming the leading cause of liver damage in Western countries (2). Accumulation of triglycerides exceeding 5% of liver weight is considered a benign response that protects against hepatic lipotoxicity due to the increased flux of fatty acids during insulin resistance (3, 4). This hepatic lipid accumulation is the hallmark of NAFLD (5). NAFLD presents as simple steatosis that may be complicated by hepatocellular damage and inflammation, namely nonalcoholic steatohepatitis (NASH) (6, 7), and may lead to cirrhosis and hepatocellular carcinoma (8). Epidemiological associations suggest that NAFLD contributes to cardiovascular disease by inducing hepatic inflammation and release of atherogenic factors (9, 10).

The interindividual predisposition to progressive NAFLD varies considerably, which is partly explained by heritability (11). The I148M variant of the *Patatin-like phospholipase domain-containing-3* gene (*PNPLA3*), which participates in lipid remodeling (12), is the major common genetic determinant of NASH,(13, 14) but other genetic factors contribute to the disease. Exome-wide association studies identified the rs58542926 C>T genetic variant of the *Transmembrane 6 superfamily member 2* gene (*TM6SF2*), which encodes the E167K aminoacidic substitution, as a determinant of hepatic triglyceride content, serum aminotransferases, and lower serum lipoproteins (15, 16). In experimental models, silencing of *TM6SF2* reduces secretion of very low density lipoproteins (VLDL) and causes a predisposition to retention of triglycerides in hepatic lipid droplets and fatty liver (15-17). In a cross-sectional study, E167K was associated with protection from myocardial infarction (16). However, serum aminotransferases do not accurately predict liver damage severity in NAFLD (18). Furthermore, although it has very recently been reported that E167K is associated with fibrosis stage in patients with NAFLD (19), the effect on the severity of hepatic lipid accumulation, and on single determinants of liver damge progression inclusing oxidative damage and necroinflammation related to the E167K variant remains undetermined. Furthermore, the effect of *TM6SF2* E167K on cardiovascular disease has not been examined in prospective studies.

In this study, we investigated whether the *TM6SF2* E167K variant–related retention of VLDL in hepatocytes is harmful. Our aim was to determine whether increased hepatic fat in *TM6SF2* E167K carriers affects the severity of steatosis, hepatocellular damage, and liver disease or cardiovascular outcomes in subjects at risk of NASH. To this end, we carried out the investigation in a large cohort who underwent liver biopsy, and evaluation for carotid atherosclerosis and in controls from the Swedish Obese Subjects (SOS) prospective cohort.

EXPERIMENTAL PROCEDURES

We studied two groups of patients. In a cross-sectional cohort of subjects who underwent a liver biopsy, we assessed the effect of *TM6SF2* E167K on liver disease and evaluated the subjects for carotid atherosclerosis. In the control group of the Swedish Obese Subjects study, we assessed the association of the *TM6SF2* E167K variant with cardiovascular risk.

LIVER BIOPSY CROSS-SECTIONAL COHORT

The cross-sectional cohort consisted of 1201 subjects enrolled in 4 European centers: Milan, Palermo, Rome (Italy), and Kuopio (Finland). The inclusion criteria were liver biopsy for suspected NASH or severe obesity (at the time of initial diagnosis) and availability of DNA samples, and clinical data. All subjects were of European descent and were consecutively enrolled at their centers of reference. Other causes of liver disease were excluded, including increased alcohol intake (men: >30 g/day; women: >20 g/day), viral and autoimmune hepatitis, hereditary hemochromatosis, alpha1-antitrypsin deficiency, and history infection with hepatitis B or hepatitis C. Patients who had decompensated cirrhosis or were taking drugs that induce steatosis were excluded. All subjects gave written informed consent. The clinical features of subjects from the liver biopsy cross-sectional cohort stratified by the recruitment center are summarized in Table S1.

Of the 1201 enrolled subjects, 581 were from the Milan center; 271 were adult patients recruited from the Metabolic Liver Diseases outpatient service between January 1999 and December 2013 and 310 underwent bariatric surgery for severe obesity between January 2006 and January 2013 at the Fondazione IRCCS Ca' Granda Ospedale Policlinico Milano. Some of these patients were previously described (20). In the Kuopio center (Northern Savo Hospital District), 197 Finnish patients undergoing bariatric surgery were recruited with analogous inclusion criteria (21). In the Palermo center, 280 adult patients were recruited from the Gastrointestinal & Liver Unit of the Palermo University Hospital (22). In the Rome center, 142 untreated children and adolescents referred to Bambino Gesù Children's Hospital were

consecutively recruited between May 2006 and November 2009. This pediatric cohort has been described (23).

Diagnosis of NASH was based on the presence of steatosis with lobular necroinflammation and ballooning or fibrosis. Disease activity was assessed according to the NAFLD activity score; fibrosis was staged according to the recommendations of the NAFLD clinical research network (6).

Carotid atherosclerosis was evaluated as described (9, 24) in 427 adult patients with histological NAFLD without severe obesity from the Milan (n=202) and Palermo (n=225) cohorts, as decribed in the results section.

THE SWEDISH OBESE SUBJECTS STUDY

The SOS study cohort has been described (25). Since bariatric surgery modifies metabolic features and cardiovascular risk (25), only the control group (subjects who did not undergo bariatric surgery) was included. A total of 1819 subjects from the SOS control group in whom the *TM6SF2* E167K variant was successfully genotyped were included.

The cardiovascular end points were fatal and nonfatal cardiovascular events (myocardial infarction or stroke). The median follow-up for cardiovascular events in the SOS control group was 14 years. The included ICD-9/ICD-10 codes were myocardial infarction (410/I21, I22), intracerebral bleeding (431/I61), cerebral artery occlusion (433, 434/I63, I65, I66), acute but nondefined stroke (bleeding or occlusion: 436/I64). Information about the end points was obtained after cross-checking of social security numbers from the SOS database with the Swedish National Patient Register, the Cause of Death Register, and the Register of the Total Population. The information was complete through December 31, 2009.

The hepatic steatosis index and NAFLD liver fat score were calculated as previously described (26). Impaired fasting glucose was defined according to WHO criteria in the SOS and the liver study cohorts.

ETHICAL APPROVAL

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and the recommendations of the Ethics Committees of the Fondazione IRCCS Ca' Granda (Milan), Bambino Gesù Children's Hospital (Rome, Milan), Palermo University Hospital (Palermo), Northern Savo Hospital District in Kuopio (Finland), and University of Gothenburg (Sweden).

GENOTYPING

The rs58542926 C>T (E167K, *TM6SF2*) and rs738409 C>G (I148M *PNPLA3*) single nucleotide polymorphisms were assessed in duplicate by TaqMan 5'-nuclease assays (Life Technologies, Carlsbad, CA). The success rate and reproducibility were >99%; random samples were confirmed by direct sequencing. The genotype distributions were in Hardy-Weinberg equilibrium.

STATISTICAL ANALYSIS

For descriptive statistics, continuous traits were summarized as means±SD. Two highly skewed variables—alanine aminotransferase (ALT) and aspartate aminotransferase (AST)—were summarized as medians and interquartile range. Categorical variables are shown as percentages. Analyses were performed by fitting data to generalized linear regression models. In particular, general linear models were fit to examine continuous traits. ALT and AST levels were log-transformed before entry into the model. Logistic regression models were fit to examine binary traits (NASH, severe fibrosis stage F3-F4, carotid plaques), and ordinal regression models were fit for ordinal traits (components of the NAFLD activity score: severity of steatosis, necroinflammation and hepatocellular ballooning, stage of fibrosis). When specified, confounding factors (including recruitment center) were included in a model.

In the SOS control group, time of progression to cardiovascular events was evaluated by Kaplan-Meier estimates of cumulative incidence rates; survival distributions were compared by Cox proportional-hazards test. Besides the *TM6SF2* E172K variant, we considered traditional cardiovascular risk factors at

baseline: age, sex, smoking status, body mass index (BMI), systolic blood pressure, glucose levels, and total cholesterol levels.

In the analysis, the *TM6SF2* E172K variant was coded in a dominant genetic model because of its relatively low allele frequency (P<0.07). The *PNPLA3* I148M variant was coded in an additive model (14).

Statistical analyses were carried out with JMP 10.0 (SAS Institute, Cary, NC) and SPSS 21.0 (IBM, Burbank, NJ). A two-sided P value <0.05 was considered statistically significant.

RESULTS

E167K TM6SF2 CARRIERS HAVE LOWER SERUM LIPID LEVELS

In the liver biopsy cross-sectional cohort, there was no significant difference in the prevalence of the E167K variant according to recruitment center (Table S1).

Demographic, anthropometric, and metabolic characteristics of the liver biopsy cross-sectional cohort stratified by *TM6SF2* genotype are summarized in Table 1. E167K carriers had lower serum lipid levels (total cholesterol and triglycerides, both P <0.05 vs. noncarriers). Features of subjects stratified by *TM6SF2* genotype subdivided according to recruitment centers (adult and pediatric liver clinics and bariatric services) are presented in Table S2.

LIVER DAMAGE DETERMINANTS IN THE LIVER BIOPSY COHORT

The clinical characteristics of the liver biopsy cross-sectional cohort stratified by severity of liver damage (absence of steatosis, simple steatosis, and NASH) are shown in Table S3. As expected, the prevalence of impaired fasting glucose or diabetes and the levels of serum triglycerides and aminotranferases increased, and HDL cholesterol levels decreased, with the severity of liver damage (P<0.05).

In the liver biopsy cohort, NASH was observed in 677 subjects (56%), and advanced fibrosis (stage F3-F4) in 128 (11%). Despite higher BMI, adult patients recruited at bariatric services (referred for severe obesity and systematically assessed for liver damage severity by biopsy independently of other risk factors) had less severe liver damage (prevalence of NASH and severe fibrosis) than those recruited at liver clinics (Table S4; p<0.001).

TM6SF2 E167K VARIANT IS INDEPENDENTLY ASSOCIATED WITH NASH AND ADVANCED FIBROSIS

The prevalence of NASH and of advanced fibrosis according to *TM6SF2* E167K status is shown in Figure 1. Variables independently associated with liver damage (NASH and advanced fibrosis) are shown in Table 2. The *TM6SF2* E167K variant was associated with a higher prevalence of NASH (odds ratio [OR]

1.84, 95% confidence interval [CI] 1.23-2.79; P=0.003 and of advanced fibrosis (OR 2.08, 95% CI 1.20-3.55; P=0.008; Fig. 1), after adjustment for age, sex, BMI, hyperglycemia, *PNPLA3* I148M, and recruitment center. NASH was also independently associated with older age, impaired fasting glucose or diabetes, and higher BMI. Advanced fibrosis was associated with older age and impaired fasting glucose or diabetes (Table 2). The association of the *TM6SF2* E167K variant with advanced fibrosis was abolished after conditioning for the presence of NASH.

The *TM6SF2* E167K variant was associated with NASH in adult patients from both liver clinics (adjusted OR 2.06, 95% CI 1.17-3.79; P=0.012) and bariatric services (adjusted OR 1.89, 95% CI 1.06-3.40; P=0.031), and with advanced fibrosis in adult patients from liver clinics (adjusted OR 2.35, 95% CI 1.23-4.23; P=0.009), but not in those from bariatric services (P=NS; only 15 subjects with advanced fibrosis were included in the analysis).

TM6SF2 E167K CARRIERS ARE MORE SUSCEPTIBLE TO LIVER DAMAGE RELATED TO NAFLD

In the liver biopsy cohort, *TM6SF2* E167K was indepedently associated with the histological severity of steatosis and of liver damage related to NAFLD, independent of age, sex, BMI, diabetes or impaired fasting glucose, *PNPLA3* I148M alleles, and recruitment center (Fig. 2). Specifically, E167K was associated with all components of the NAFLD activity score (steatosis grade, P<0.001; lobular necroinflammation, P=0.040; hepatocellular ballooning, P=0.036; and fibrosis stage, P=0.022; shown in Fig. 2).

TM6SF2 E167K VARIANT PROTECTS FROM CAROTID ATHEROSCLEROSIS IN NAFLD

Clinical features of a subgroup of 427 patients from the liver biopsy cohort who were evaluated for carotid atherosclerosis are presented in Table 3. Carotid plaques were associated with risk factors for atherosclerosis, including older age, BMI, impaired fasting glucose or diabetes, hypertension, and current cigarette smoking (P<0.05; Table 3). Because of collinearity with E167K, total cholesterol and LDL

cholesterol levels were not fitted in this model. Athough patients with NASH had a higher prevalence of plaques (nominal P=0.049), carriers of E167K had a lower prevalence of plaques (P=0.031; Fig. 1, Table S4). This protective effect of E167K against carotid atherosclerosis (OR 0.48, 95% CI 0.25-0.94; P=0.031) remained significant after adjustment for risk factors and NASH (Table 3).

TM6SF2 E167K CARRIERS WITH SEVERE OBESITY ARE PROTECTED FROM CARDIOVASCULAR EVENTS

In the SOS control group, 294 (16%) subjects were carriers of the *TM6SF2* E167K variant (P=0.02 vs. carriers in the liver biopsy cohort). Carriers of the *TM6SF2* 167K allele had lower baseline levels of serum total cholesterol, non-HDL cholesterol, and apolipoprotein B, as well as higher levels of serum AST and ALT (Table 4).

The effect of E167K on cardiovascular events is shown in Figure 3. During the follow-up, 21 (7%) carriers of the E167K variant vs. 185 (12%) noncarriers developed cardiovascular disease (hazard ratio 0.61, 95% CI 0.39-0.95, P=0.030; Fig. 3, and Table 5). However, the association between E167K and cardiovascular disease disappeared after serum total cholesterol level was included in the model (P=0.091; Table 5).

DISCUSSION

Although accumulation of triglycerides in the liver has been considered a benign and protective response to insulin resistance (4), NAFLD has been proposed as an independent risk factor for cardiovascular disease (9, 10, 27). In this study, we tested whether the *TM6SF2* E167K genetic variant, a determinant of triglycerides accumulation in the liver (15, 16), increases risk of hepatic inflammation and fibrosis while simultaneously influencing cardiovascular risk. We found that carriers of the E167K variant are protected from cardiovascular disease at a cost of higher liver steatosis, inflammation, and fibrosis.

First, we examined the effect of fat accumulation conferred by the *TM6SF2* E167K variant in a large cohort of individuals of European descent who underwent liver biopsy for suspected NASH. In this cohort, E167K was associated with NASH and advanced fibrosis and thus conferred a high risk of liver-related complications. We showed for the first time that E167K is associated with NASH, hepatocellular ballooning and necroinflammation. Moreover, we show a robust association between this variant and histological severity of steatosis and besides that with the full spectrum of fibrosis severity. In addition, the association between the E167K variant and advanced fibrosis was abolished after conditioning for NASH, suggesting that fibrosis progression is mediated by hepatocellular lipid retention with consequent oxidative damage and inflammation in carriers of the genetic variant. The *TM6SF2* E167K aminoacidic substitution results in an unstable protein that reduces VLDL-mediated cellular export of neutral fat (triglycerides and cholesterol esters) (15, 16). The increased hepatic inflammation and fibrosis in E167K carriers suggests that accumulation of triglycerides in hepatocytes directly favors NASH and fibrosis.

Previous studies highlighted the association of the severity hepatic triglycerides accumulation with liver damage in NAFLD (28). In addition, the *PNPLA3* I148M variant, the major genetic determinant of NASH, impairs both triglyceride remodeling in lipid droplets and export of triglycerides to VLDL in hepatocytes (12, 29). A possible alternative mechanism may be related to the toxicity of excessive hepatocellular cholesterol and the consequent mitochondrial damage in carriers of the E167K variant (30, 31). Nevertheless, the deleterious effect of impaired ability to secrete VLDL is supported by the

association between progressive liver disease and rare Apolipoprotein B mutations that cause VLDL retention (32).

In a recent cross-sectional study, the E167K variant was linked to lower circulating lipoprotein levels and protection against myocardial infarction (16). In line with these findings, carriers of the E167K variant had a lower prevalence of carotid plaques in a subgroup of the liver biopsy cross-sectional cohort. We extended these findings in a large prospective cohort of morbidly obese individuals at high risk of cardiovascular disease with long-term follow-up. In this cohort, we showed that E167K carriers have lower serum lipids and are protected against cardiovascular disease. After adjustment for cholesterol levels, however, the E167K variant was no longer associated with cardiovascular outcomes. Evidently, the protective effect of E167K reflects the reduced circulating levels of atherogenic lipoproteins. These findings show that increased susceptibility to liver disease is accompanied by protection against cardiovascular disease in carriers of the *TM6SF2* E167K variant in individuals at risk for NASH due to insulin resistance and/or severe obesity.

The association of E167K with NASH and advanced fibrosis contradicts the notion that long-term hepatic storage of triglycerides due to impaired export of VLDL is benign (32). The association between the *NCAN* locus on chromosome 19 and NAFLD severity (33, 34), and a very recent communication reporting the association of the E167K variant with moderate/severe hepatic fibrosis (19), support the association between *TM6SF2* and NAFLD severity. In fact, the E167K is the causal variant explaining the association of the *NCAN* locus with hepatic triglyceride content and lipid levels (15, 16).

These results may have implications for the treatment of severe hypercholesterolemia with drugs targeting hepatic VLDL secretion, which lower serum lipids by up to 25–50% (35, 36). We found that the *TM6SF2* E167K variant, which similarly interferes with VLDL secretion, predisposes to severe liver disease by decreasing lipid levels by 5–10%, consistent with previous reports (15, 16). Thus, pharmacologic inhibition of VLDL secretion will possibly result, in the long term, in severe liver disease. In support of this possibility, liver enzymes are frequently elevated in parallel with a substantial rise in hepatic fat in patients treated with inhibitors of VLDL secretion in clinical trials (35, 36).

A limitation of this study is that liver damage and cardiovascular risk were separately evaluated in two different cohorts of European subjects, recruited from both hepatology and bariatric services. However, increased hepatic fat and protection against cardiovascular disease have been observed in carriers of the E167K variant in heterogeneous populations (15, 16). Furthermore, we showed that the E167K variant was protective against carotid plaques — a strong predictor of atherosclerosis burden and cardiovascular risk (37)— in a subgroup of patients with NAFLD. On the other hand, levels of ALT, an indirect index of liver damage, were higher in E167K carriers in the SOS study. Strengths of the study include the direct assessment with liver biopsy of liver damage and fibrosis in a large number of individuals and the prospective evaluation of all cardiovascular events in a high-risk cohort (25).

In conclusion, we found that the *TM6SF2* E172K variant increases susceptibility to NASH and liver fibrosis, but protects against cardiovascular events. Our findings suggest that inhibition of VLDL secretion from the liver protects against cardiovascular disease, but at the cost of an increased risk of severe liver disease.

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FIGURE LEGENDS

Figure 1. In the Liver Biopsy Cross-Sectional Cohort, Carriers of the *TM6SF2* E167K Variant Have More Hepatic Inflammation and Fibrosis but Less Vascular Atherosclerosis.

Prevalence of nonalcoholic steatohepatitis (NASH), advanced fibrosis (stage F3-F4), and carotid plaques according to the *TM6SF2* E167K genotype (dominant model) in 1201 subjects who underwent liver biopsy for suspected NASH. *Adjusted for age, sex, BMI, presence of impaired fasting glucose or diabetes, *PNPLA3* I148M alleles, and recruitment center; †adjusted for age, sex, BMI, presence of impaired fasting glucose or diabetes, arterial hypertension, smoking status, and the presence of NASH. ‡Estimation of carotid plaques by ecocolordoppler of the common carotid arteries was performed in a subgroup of 427 subjects from the liver biopsy cross-sectional cohort with histologically confirmed NAFLD. Specifically, 356 individuals had E167E genotype, whereas 71 were carriers of the 167K allele.

Figure 2. In the Liver Biopsy Cross-Sectional Cohort, the *TM6SF2* E167K Variant Is Associated with the Severity of Histological Damage, As Evaluated by the Different Components of the NAFLD Activity Score and with Hepatic Fibrosis Stage.

Non-carriers: homozygotes for the *TM6SF2* 167E allele E167E genotype; Carriers: carriers of *TM6SF2* 167E allele: (E167K and K167K genotypes). The association between the *TM6SF2* E167K variant and the components of the NAFLD activity score have been tested by multivariate ordinal regression analysis. The analysis has been also adjusted for age, gender, BMI, presence of diabetes or impaired fasting glucose, *PNPLA3* I148M alleles, and recruitment center. BMI, body-mass index; adj p: adjusted p value.

Figure 3. In the Swedish Obese Subjects Study, Carriers of the *TM6SF2* 167K Allele Have a Lower Incidence of Fatal and NonFatal Cardiovascular Events.

Cumulative incidence of fatal and nonfatal cardiovascular diseases (myocardial infarction and stroke, whichever came first) in the SOS control group across *TM6SF2* rs58542926 genotypes. A total of 185 (12%) homozygotes for the 167E allele developed cardiovascular events, compared to 21 (7%) E167K and K167K subjects. A Cox proportional hazard model was fit to compare survival distributions between the two genotype groups adjusting for risk factors age, sex, smoking status, BMI, systolic blood pressure, and glucose levels. SOS, Swedish obese subjects; CVD, cardiovascular disease; HR, hazard ratio; CI, confidence interval.

TABLES

Table 1. Demographic, anthropometric and clinical characteristics of the liver biopsy cross-sectional cohort stratified by carriage of the *TM6SF2* 167K allele.

	TM6SF2 167K allele		
	Noncarriers	Carriers	
Characteristic	n=1044 (87%)	n=157 (13%)	P value*
Age, years	42±16	42±16	0.77
Sex, female	505 (48)	70 (45)	0.54
BMI, kg/m^2	34.3±9	33.9±10	0.93
IFG/diabetes	289 (28)	37 (24)	0.31
Total cholesterol, mmol/l	4.6±1.1	4.4±1.0	0.038
LDL cholesterol, mmol/l	3.0±1.0	2.9±0.9	0.087
HDL cholesterol, mmol/l	1.2±0.4	1.2±0.3	0.91
Triglycerides, mmol/l	1.6±0.9	1.4±0.8	0.009
ALT, IU/l	44 {25-71}	46 {28-72}	0.45
AST, IU/l	30 {21-44}	30 {21-43}	0.78

Values are mean ± SD, or number (%).

*Comparisons were made by fitting data to generalized linear models. P values were adjusted for age, gender, BMI, impaired fasting glucose or diabetes, and recruitment center, except when each of these covariates was under consideration. Serum lipid levels were further adjusted for treatment with statins.

IFG, impaired fasting glucose; LDL, low-density lipoprotein cholesterol (estimated with Friedewald's formula); HDL, high-density lipoprotein cholesterol.

Table 2. Multiple logistic regression analysis of variables associated with the severity of liver disease in the liver biopsy cross-sectional cohort.

	NASH			Fibrosis F3-F4		
Characteristic	OR	95% CI	P value*	OR	95% CI	P value*
Age, years	1.02	1.00-1.03	0.006	1.06	1.05-1.10	<0.001
Sex, female	0.80	0.59-1.09	0.16	1.09	0.69-1.72	0.70
IFG/diabetes	1.98	1.42-2.76	<0.001	2.84	1.79-4.56	<0.001
BMI, kg/m^2	1.08	1.05-1.11	<0.001	1.04	1.00-1.09	0.062
PNPLA3 I148M	1.65	1.35-2.01	<0.001	1.54	1.16-2.07	0.003
TM6SF2 E167K	1.84	1.23-2.79	0.003	2.08	1.20-3.55	0.008

IFG, impaired fasting glucose. *Comparisons were made by fitting data to ordinal regression models considering as independent variables: age, gender, impaired fasting glucose or diabetes, BMI, *PNPLA3* I148M alleles, presence of the *TM6SF2* E167K variant, and recruitment center.

Table 3. Clinical characteristics in a subgroup (n=427) of the liver biopsy cross-sectional cohort stratified by the presence of carotid plaques, and variables independently associated with the presence of plaques.

	Carotid plaques			
	Absent	Present		P
Characteristic	n=271 (63%)	n=156 (37%)	OR (95% CI)	value
Sex, female	67 (27)	51 (33)	1.33 (0.80-2.22)	0.27
Age, years	45±12	55±11	1.04 (1.02-1.05)	<0.001
BMI, kg/m^2	28.3±4.6	29.3±4.4	0.93 (0.90-0.97)	<0.001
IFG/Diabetes	36 (13)	53 (34)	2.20 (1.28-3.81)	0.004
HDL, mmol/l	1.3±0.4	1.3±0.4	0.99 (0.98-1.00)	0.37
Hypertension, yes	61 (23)	86 (56)	3.29 (2.03-5.35)	<0.001
Smoking, yes	43 (16)	39 (26)	2.06 (1.19-3.61)	0.010
NASH	174 (64)	115 (73)	1.46 (0.86-2.49)	0.16
TM6SF2 167K allele carriers	53 (20)	18 (11)	0.49 (0.25-0.94)	0.031

Estimation of carotid plaques by ecocolordoppler of the common carotid arteries was performed in a subgroup of 427 subjects from the liver biopsy cross-sectional cohort with histologically confirmed NAFLD.

Values are mean ± SD, median {IQR}, or number (%). A multiple logistic regression model was fit to identify variables independently associated with carotid plaques. Levels of total and LDL cholesterol and treatment with statins were not fitted in this model, owing to collinearity with the E167K variant.

IFG, impaired fasting glucose; HDL, high-density lipoprotein cholesterol.

Table 4. Demographic, anthropometric and clinical characteristics of the SOS control group in carriers and non-carriers of the *TM6SF2* 167K allele.

	TM6SF2 167K allele		
	Noncarriers	Carriers	P value*
Characteristic	n=1525	294[†]	
Sex, female	1073 (70%)	n=207 (70%)	0.99
Age, years	49±6	49±7	0.55
BMI, kg/m^2	40±5	40±5	0.92
Systolic blood pressure, mmHg	138±18	137±17	0.65
Blood glucose, per mmol/l	4.9±1.7	5.1±2.1	0.04
Insulin, mU/l	18±12	19±11	0.40
HOMA-IR, U	4.2±4.0	4.5±4.5	0.14
IFG/Diabetes, yes	322 (21%)	64 (22%)	0.86
ALT, IU/l	46 {35-63}	51 {38-76}	<0.001
AST, IU/l	35 {28-44}	37 {29-49}	0.007
AST / ALT ratio	0.73 {0.61-0.91}	0.71 {0.59-0.84}	<0.001
HSI, >36	1519 (99)	294 (100)	0.27
NAFLD liver fat score, >-0.640	1229 (81)	246 (84)	0.25
Total cholesterol, mmol/l	5.6±1.1	5.4±1.0	<0.001
HDL cholesterol, mmol/l	1.3±0.3	1.3±0.3	0.48
Triglycerides, mmol/l	2.1±1.5	1.8±1.0	0.006
Non-HDL cholesterol, mmol/l	4.3±1.1	4.0±1.1	<0.001
APOB, g/l	1.24±0.3	1.19±0.3	0.003
Smoking, n (%)	320 (21)	51 (17)	0.18

Values mean ± SD, median {IQR}, or number (% value). Comparisons were made by fitting data to generalized linear models. *P values were adjusted for the following covariates: age, sex, BMI, and diabetes status, except when each of these covariates was under consideration.

†274 subjects were heterozygous and 20 homozygous for the E167K variant.

HSI: hepatic steatosis index; HOMA-IR, homeostasis model assessment for insulin resistance; IFG: impaired fasting glucose; HDL, high-density lipoprotein; APOB, apolipoprotein B.

Table 5. Multivariable Cox proportional hazards model for cardiovascular events in 1819 subjects from the SOS control group.

	Hazard Ratio (95% CI)	P value
TM6SF2 K allele carriers, yes/no	0.60 (0.38-0.95)	0.028
TM6SF2 K allele carriers, yes/no	0.62 (0.40-0.98)	0.041
Female gender, yes/no	0.44 (0.33-0.58)	<0.001
Age, per 10 years	1.94 (1.52-2.48)	<0.001
Smoking, yes/no	2.15 (1.59-2.89)	<0.001
BMI, per kg/m^2	1.01 (0.98-1.04)	0.481
Systolic blood pressure, per 10 mm Hg	1.14 (1.06-1.23)	<0.001
IFG/Diabetes, yes/no	2.27 (1.70-3.01)	<0.001
TM6SF2 K allele carriers, yes/no	0.70 (0.44-1.10)	0.120
Female gender, yes/no	0.44 (0.33-0.59)	<0.001
Age, per 10 years	1.88 (1.47-2.42)	<0.001
Smoking, yes/no	1.98 (1.46-2.68)	<0.001
BMI, per kg/m^2	1.01 (0.98-1.05)	0.431
Systolic blood pressure, per 10 mmHg	1.14 (1.06-1.23)	0.001
IFG/Diabetes, yes/no	2.19 (1.65-2.92)	<0.001
Total cholesterol, per mmol/l	1.30 (1.16-1.46)	<0.001

First the TM6F2K allele was included in the analyses, and then traditional risk factors for cardiovascular disease were included. Total cholesterol was included last, owing to collinearity with the genetic variant. Indeed the genetic variant was an independent predictor of protection against cardiovascular disease until the cholesterol was entered in the model.

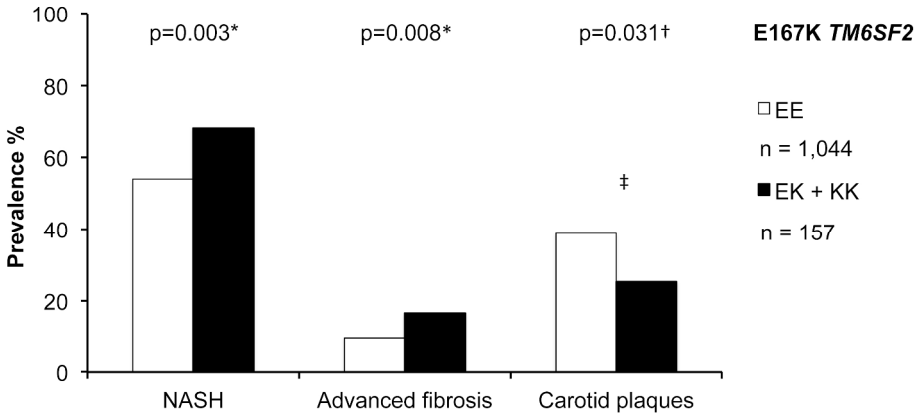

Figure 1. In the Liver Biopsy Cross-Sectional Cohort, Carriers of the TM6SF2 E167K Variant Have More Hepatic Inflammation and Fibrosis but Less Vascular Atherosclerosis.
Prevalence of nonalcoholic steatohepatitis (NASH), advanced fibrosis (stage F3-F4), and carotid plaques according to the TM6SF2 E167K genotype (dominant model) in 1201 subjects who underwent liver biopsy for suspected NASH. *Adjusted for age, sex, BMI, presence of impaired fasting glucose or diabetes, PNPLA3 I148M alleles, and recruitment center; †adjusted for age, sex, BMI, presence of impaired fasting glucose or diabetes, arterial hypertension, smoking status, and the presence of NASH. ‡Estimation of carotid plaques by ecocolordoppler of the common carotid arteries was performed in a subgroup of 427 subjects from the liver biopsy cross-sectional cohort with histologically confirmed NAFLD. Specifically, 356 individuals had E167E genotype, whereas 71 were carriers of the 167K allele.
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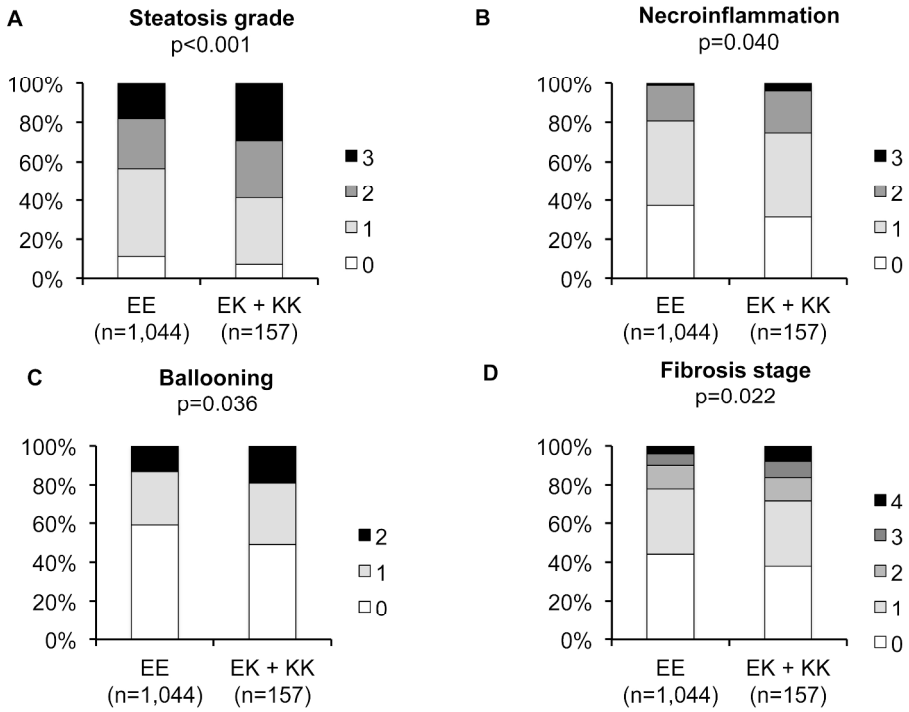

Figure 2. In the Liver Biopsy Cross-Sectional Cohort, the TM6SF2 E167K Variant Is Associated with the Severity of Histological Damage, As Evaluated by the Different Components of the NAFLD Activity Score and with Hepatic Fibrosis Stage.
Non-carriers: homozygotes for the TM6SF2 167E allele E167E genotype; Carriers: carriers of TM6SF2 167E allele: (E167K and K167K genotypes). The association between the TM6SF2 E167K variant and the components of the NAFLD activity score have been tested by multivariate ordinal regression analysis. The analysis has been also adjusted for age, gender, BMI, presence of diabetes or impaired fasting glucose, PNPLA3 I148M alleles, and recruitment center. BMI, body-mass index; adj p: adjusted p value.
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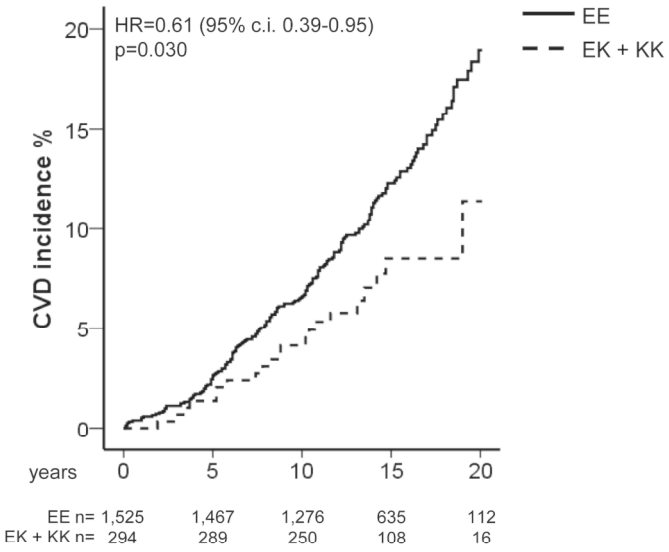

Figure 3. In the Swedish Obese Subjects Study, Carriers of the TM6SF2 167K Allele Have a Lower Incidence of Fatal and NonFatal Cardiovascular Events.

Cumulative incidence of fatal and nonfatal cardiovascular diseases (myocardial infarction and stroke, whichever came first) in the SOS control group across TM6SF2 rs58542926 genotypes. A total of 185 (12%) homozygotes for the 167E allele developed cardiovascular events, compared to 21 (7%) E167K and K167K subjects. A Cox proportional hazard model was fit to compare survival distributions between the two genotype groups adjusting for risk factors age, sex, smoking status, BMI, systolic blood pressure, and glucose levels. SOS, Swedish obese subjects; CVD, cardiovascular disease; HR, hazard ratio; CI, confidence interval.

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SUPPLEMENTARY TABLES

Table S1. Demographic, anthropometric, and clinical characteristics of the liver biopsy cross-sectional cohort (n=1201) stratified by recruitment center.

Characteristic	Liver Clinic			Bariatric surgery	
	Adult		Pediatric		
	Milan (n=271)	Palermo (n=280)	Rome (n=142)	Milan (n=310)	Kuopio (n=198)
Age, years	50±12	46±13	10±3	43±11	48±9
Sex, female	50 (18)	96 (35)	54 (38)	244 (79)	131 (66)
BMI, kg/m^2	27.3±3	30.0±5	25.6±5	41.7±8	44.3±6
IFG/diabetes	50 (19)	63 (23)	70 (49)	56 (18)	87 (44)
Cholesterol, mmol/l	4.7±1.0	4.9±1.1	3.9±0.5	5.0±1.1	3.7±0.9
LDL, mmol/l	3.2±1.1	3.3±1.0	2.6±0.4	3.3±0.9	2.3±0.8
HDL, mmol/l	1.2±0.4	1.3±0.4	1.1±0.2	1.3±0.4	1.0±0.3
Triglycerides, mmol/l	1.7±0.9	1.6±0.9	1.3±0.8	1.6±0.9	1.6±0.7
ALT IU/l	46 {30-69}	67 {48-97}	70 {52-89}	21 {16-32}	39 {26-55}
NAFLD	271 (100)	180 (100)	142 (100)	267 (86)	129 (65)
NASH	122 (44)	246 (88)	106 (75)	134 (43)	69 (35)
Fibrosis F3-F4	43 (16)	62 (22)	8 (6)	8 (3)	7 (4)
PNPLA3 I148M					
II	99 (36)	101 (36)	62 (43)	151 (49)	127 (64)
IM	122 (45)	124 (44)	58 (41)	130 (42)	58 (29)
MM	50 (19)	55 (20)	22 (16)	29 (9)	13 (7)
*TM6SF2*E167K					
EE	231 (85)	238 (85)	126 (89)	EE 275 (89)	174 (88)
EK	38 (14)	42 (15)	16 (11)	EK 35 (11)	24 (12)
KK	2 (1)	0	0	0	0

Values are mean ± SD, median {IQR}, or number (%).

*LDL levels were estimated according to the Friedewald's formula.

IFG, impaired fasting glucose; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol (estimated with Friedewald's formula).

P=0.69 for the prevalence of the E167K variant across recruitment centers, and P=0.20 for the prevalence of of the E167K variant between liver clinics (Milan, Palermo, Rome) and bariatric series (Milan, Kuopio).

Table S2. Demographic, anthropometric and clinical characteristics of the liver biopsy cross-sectional cohort according to the modality of recruitment stratified by carriage of the *TM6SF2* 167K allele.

	Liver clinic, adults, n=551 (46)			**Liver clinic, pediatric, n=142 (12)**			**Bariatric services, n=508 (42)**		
TM6SF2 167K allele	Noncarriers n=469 (85)	Carriers n=82 (15)	P value*	Noncarriers n=126 (89)	Carriers n=16 (11)	P value*	Noncarriers n=449 (88)	Carriers n=59 (12)	P value*
Age, years	48±13	47±13	NS	10±2	10±3	NS	45±10	42±11	NS
Sex, female	130 (28)	16 (20)	NS	46 (36)	8 (50)	NS	329 (72)	46 (78)	NS
BMI, kg/m^2	28.6±5	28.5±4	NS	25.7±5	25.2±5	NS	42.6±7	43.6±7	NS
IFG/diabetes	104 (22)	9 (11)	NS	62 (49)	8 (50)	NS	123 (27)	20 (34)	NS
Total cholesterol, mmol/l	4.8±1.1	4.6±1.0	NS	3.9±0.6	3.9±0.4	NS	4.5±1.2	4.2±1.0	NS
LDL cholesterol, mmol/l	3.2±1.1	3.1±0.9	NS	2.6±0.5	2.6±0.3	NS	2.9±1.0	2.7±0.9	NS
HDL cholesterol, mmol/l	1.2±0.4	1.2±0.3	NS	1.1±0.2	1.1±0.2	NS	1.2±0.4	1.2±0.3	NS
Triglycerides, mmol/l	1.7±0.9	1.4±0.8	0.007	1.3±0.8	1.2±0.7	NS	1.6±0.8	1.5±0.9	NS
ALT, IU/l	55 {36-53}	58 {39-80}	NS	70{51-88}	79 {54-90}	NS	27 {18-44}	27 {18-38}	NS
AST, IU/l	24 {20-33}	27{20-36}	NS	48 {39-66}	56 {43-68}	NS	21 {16-28}	21 {16-27}	NS

Values are mean ± SD, or number (%). NS: not significant.

*Comparisons were made by fitting data to generalized linear models. P values were adjusted for age, gender, BMI, impaired fasting glucose or diabetes, and recruitment centre, except when each of these covariates was under consideration. Serum lipid levels were further adjusted for treatment with statins.

IFG, impaired fasting glucose; LDL, low-density lipoprotein cholesterol (estimated with Friedewald's formula); HDL, high-density lipoprotein cholesterol.

Table S3. Demographic, anthropometric and clinical characteristics of the liver biopsy cross-sectional cohort stratified by the severity of liver disease.

Characteristic	No steatosis n=112 (9%)	Simple steatosis n=412 (34%)	Steatohepatitis n=677 (57%)	P value
Age, years	45±10	42±14	41±17	0.050
Sex, F	87 (76)	197 (48)	294 (43)	0.32
BMI, kg/m^2	41.3±8	33.5±9	33.4±9	<0.001
IFG/diabetes	29 (25)	75 (18)	224 (33)	<0.001
Total cholesterol, mmol/l	4.2±1.1	5.3±1.2	5.0±1.2	0.12
LDL, mmol/l	2.6±0.9	3.4±1.0	3.2±1.0	0.085
HDL, mmol/l	1.2±0.4	1.3±0.4	1.2±0.4	0.018
Triglycerides, mmol/l	1.4±0.6	1.5±0.8	1.6±0.9	0.002
ALT, IU/l	25 {15-39}	33 {20-54}	56 {35-84}	<0.001
AST, IU/l	21 {15-29}	24 {18-34}	36 {25-51}	<0.001

Data are shown as mean ± SD, median {IQR}, or number (% value). *Comparisons were made by fitting data to generalized linear models assuming a trend across categories of liver damage. P values were adjusted for the following covariates: age, gender, BMI, IFG/diabetes, and recruitment center, except when each of these covariates was under consideration.

IFG, impaired fasting glucose; Chol, cholesterol; LDL, low-density lipoprotein cholesterol (estimated with Friedewald's formula); HDL, high-density lipoprotein cholesterol.

Table S4. Liver damage severity stratified by the recruitment criteria (liver clinics vs. bariatric services) in adult patients from the liver biopsy cross-sectional cohort.

	Liver clinics, n=551	Bariatric services, n=508	P value*
NASH	369 (67)	202 (35)	<0.001
Fibrosis F3-F4	105 (19)	15 (3)	<0.001

Data are shown as number (% value). NASH: nonalcoholic steatohepatitis. P values were adjusted for the following covariates: age, gender, BMI, IFG/diabetes

Table S5. Prevalence of carotid plaques in 427 patients of the liver biopsy cross-sectional cohort who underwent evaluation of carotid atherosclerosis stratified by carriage of the *TM6SF2* 167K allele and presence of NASH.

	Prevalence of carotid plaques, (%)	
	EE	EK + KK
Overall, n=427	138/356 (39)	18/71 (25)
Simple steatosis, n=137	37/119 (31)	4/18 (22)
NASH, n=290	101/237 (43)	14/53 (26)