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VIRAL HEPATITIS

TM6SF2 rs58542926 is not associated with steatosis and fibrosis in large cohort of patients with genotype 1 chronic hepatitis C

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Abstract

Background & Aims: We tested the putative association of the rs58542926 variant of *TM6SF2*, a recently described genetic determinant of nonalcoholic fatty liver disease, with steatosis and fibrosis in genotype 1(G1) chronic hepatitis C(CHC) patients. *Methods:* A total of 694 consecutively biopsied Caucasian G1 CHC patients were genotyped for *TM6SF2* rs58542926, *IL28B* rs12979860 and *PNPLA3* rs738409. Steatosis was classified as absent (<5%), mild-moderate(5–29%) and severe(≥30%), Fibrosis was considered severe if=F3-F4. *Results:* Carriers of *TM6SF2* rs58542926 (6.3% of patients) exhibited lower serum levels of cholesterol ($P = 0.04$) and triglycerides ($P = 0.01$), but a similar distribution of steatosis severity ($P = 0.63$), compared to noncarriers. Prevalence and severity of steatosis were reduced in *IL28B* C allele carriers ($P = 0.005$) and elevated in *PNPLA3* G allele carriers ($P < 0.001$). After adjustment for age, gender, body mass index and homoeostasis model assessment score, steatosis severity was independently associated with *IL28B* rs12979860 (odds ratio [OR] 0.69, 95% confidence interval [CI] 0.55–0.86, $P = 0.001$) and *PNPLA3* rs738409 (OR 1.84, 95% CI 1.46–2.83, $P < 0.001$), but not *TM6SF2* rs58542926 (OR 1.48, 95% CI 0.82–2.69, $P = 0.19$). Variants of *TM6SF2* (30.9% vs. 25%, $P = 0.40$), *IL28B* and *PNPLA3* were not directly associated with fibrosis severity, although variants of *IL28B* and *PNPLA3* promoted steatosis (OR 1.36, 95% CI 1.06–1.75, $P = 0.01$) that in turn is associated with severe fibrosis. *Conclusions:* In G1 CHC patients, *TM6SF2* rs58542926 does not affect the histological severity of liver damage. However, *IL28B* rs12979860 and *PNPLA3* rs738409 modify steatosis.

Keywords
CHC – IL28B – PNPLA3 – steatosis – TM6SF2

The estimated global prevalence of hepatitis C virus (HCV) infection is 2.2%, corresponding to about 130 million HCV-positive persons worldwide, most of whom are chronically infected (1). HCV is one of the main causes of cirrhosis, hepatocellular carcinoma and liver transplantation in Western countries. The recent development of interferon-free therapeutic regimens to treat HCV would allow for the eradication of infection in more than 90% of treated patients and a strong improvement in the natural history of their liver disease (2). However, the impossibility of treating all infected patients (because of cost) and the presence of numerous cofactors that accelerate the progression and complications of HCV-associated liver disease have prompted clinical research to understand the modifiers of liver damage.

In this complex landscape, hepatic steatosis and some of its genetic determinants exert key roles as modifiers of liver disease severity in patients with genotype 1 (G1) chronic hepatitis C (CHC). Specifically, steatosis has

Abbreviations
CHC, chronic hepatitis C; G1, genotype 1.

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Key points

- The *TM6SF2* rs58542926 gene variant has been associated with the presence and histological severity of nonalcoholic fatty liver disease.
- Previously reported preliminary data in hepatitis C virus-infected patients suggested that *TM6SF2* rs58542926 modulates steatosis.
- In this study of the largest cohort of genotype 1 chronic hepatitis C patients, we found that the *TM6SF2* rs58542926 gene variant is not linked to the presence or severity of fatty liver or fibrosis.
- We confirmed the association of variants of *PNPLA3* and *IL28B* in modifying steatosis.

been associated with an increased severity of liver fibrosis (3). It also has emerged as a predictor of fibrosis progression (4) and hepatocellular carcinoma occurrence (5). Variants of two genes, *PNPLA3* and *IL28B*, have been reported to affect the prevalence and severity of steatosis, the severity of fibrosis and the prognosis of liver disease in patients with CHC (6–16). Specifically, the *PNPLA3* rs738409 and *IL28B* rs12979860 variants are the strongest known genetic risk factors for occurrence of nonalcoholic fatty liver disease (NAFLD) (6) and for response to interferon/ribavirin therapy in CHC (7) respectively.

A recent exome-wide study identified the rs58542926 variant of *TM6SF2*, a gene that modulates lipid export from the liver, as a new genetic determinant of NAFLD (17). This variant is a nonsynonymous C>T polymorphism. Recent studies by others and us have demonstrated that this variant increases the risk that NAFLD patients will develop liver fibrosis and steatohepatitis (18–20). Only two studies have examined the effects of the *TMS6SF2* variant in CHC patients (21,22). However, the inclusion of CHC patients with different HCV genotypes complicates the interpretation of these studies. Therefore, a large study of this variant in CHC patients with a single HCV genotype has been necessary. The aim of this study was to assess the putative association between the *TM6SF2* polymorphism and histological steatosis, as well as the impact on the severity of liver fibrosis in a large cohort of G1 CHC patients.

Patients and methods

This study cohort comprised 694 CHC patients who were consecutively enrolled at the Gastrointestinal and Liver Unit of the University Hospital Palermo, Italy. The following inclusion criteria were applied: (a) diagnosis of G1 CHC based on HCV serology and viral RNA, (b) histological diagnosis of CHC by liver biopsy, (c) self-reported alcohol consumption of less than 20 g/day in the last 12 months, as evaluated by a questionnaire and (d) availability of DNA for genotyping. Patients were excluded from this study if they were co-infected with either the hepatitis B virus or HIV.

This study was carried out in accordance with the principles of the Helsinki Declaration and its appendices, as well as with local and national laws. Approval was obtained from the AOUP Paolo Giaccone of Palermo's Internal Review Board and its Ethics Committee. Written informed consent was obtained from all patients.

Clinical and laboratory evaluation

Clinical and anthropometric data were collected at the time of liver biopsy. Patients with a body mass index (BMI) of 30 kg/m^2 or greater were classified as obese. Impaired glucose tolerance and type 2 diabetes mellitus (T2DM) were diagnosed on the basis of the revised criteria of the American Diabetes Association (fasting blood glucose of 100–125.9 mg/dl for impaired glucose tolerance or ≥126 mg/dl for T2DM) (23). In patients with a previous diagnosis of T2DM, current therapy with insulin or oral hypoglycaemic agents was recorded.

A 12-h overnight fasting blood sample was drawn at the time of biopsy to determine the serum levels of ALT, total cholesterol (TC), triglycerides (TG), plasma glucose, insulin and platelet count. Insulin resistance (IR) was determined according to the homoeostasis model assessment (HOMA) method (24): Insulin resistance (HOMA-IR) = Fasting insulin (μU/ml) × Fasting blood glucose (mmol/L)/22.5.

Genotyping

DNA was extracted from peripheral blood collected at the time of enrolment in all patients. Genotyping for *TM6SF2* (rs58542926), *PNPLA3* (rs738409) and *IL28B* (rs12979860) was carried out by using the TaqMan SNP genotyping allelic discrimination method (Applied Biosystems, Foster City, CA, USA). Genotyping calls were made by using the SDS software package (v.1.3.0, ABI Prism 7500, Foster City, CA, USA). Genotyping was conducted in a blinded fashion relative to patient characteristics.

Histopathology

A liver biopsy was performed for all patients. Liver histology was used to determine the extents of steatosis, hepatic necroinflammation and fibrosis. Steatosis was graded as absent (<5%), mild-moderate (5–29%) or severe (≥30%). Liver fibrosis and necroinflammatory activity were classified according to the Scheuer score (25).

Statistical analysis

Our study had a power of more than 80% for detecting an odds ratio (OR) of 1.5 for steatosis for the *TM6SF2*

rs58542926 variant, with a significance of 5%. Continuous variables were summarized as the mean ± SD, and categorical variables as the frequency and percentage. The *t*- and χ^2 tests were used where appropriate. Multiple ordinal regressions were used to assess variables associated with the severity of steatosis. Multiple logistic regression models were used to assess the factors associated with severe fibrosis. ORs and 95% confidence intervals (CIs) are reported.

As candidate risk factors, we used gender, age, BMI, HOMA score, *TM6SF2* rs58542926 genotype, *PNPLA3* rs738409 genotype and *IL28B* rs12979860 genotype. In the analysis, the *TM6SF2* E172K variant was coded in a dominant genetic model because of its relatively low allele frequency (19,20). The *PNPLA3* and *IL28B* variants were coded in an additive model. Analyses were performed by using the SPSS software package.

Results

Patient features and histology

Baseline characteristics of the 694 patients are shown in Table S1. The mean age was 53 years, and 54% of patients were male. Obesity and T2DM each were observed in about 13% of patients. Mean TC and TG levels were within the normal range. The mean HOMA value was 3.37. One-third of patients had severe fibrosis. Steatosis was observed in about half of cases, and 17% had high-grade steatosis.

Distributions of the *IL28B* rs12979860 and *PNPLA3* rs738409 genotypes are reported in Table S1. For the *TM6SF2* rs58542926 variant, 93.7% of patients were homozygous for the C allele, and 6.3% of cases carried the T variant. Only one patient was homozygous for the T allele. Genetic frequencies were in Hardy–Weinberg equilibrium. In our cohort, the metabolic profile differed according to the *TM6SF2* genotype (Table 1). Patients carrying the rs58542926 variant were characterized by lower serum levels of TC ($P = 0.04$) and TG ($P = 0.01$) compared to noncarriers.

Table 1. Characteristics of 694 patients with genotype 1 chronic hepatitis C, according to *TM6FS2* rs58542926 genotype

Variable	TM6SF2 CC $n = 650$ (93.6)	TM6SF2 TC/TT $n = 44$ (6.4)	*P* value
Age (yrs)	53.7 ± 11.9	52.9 ± 11.7	0.65
Male gender (%)	53.8	61.3	0.33
Body mass index (kg/m^2)	26.6 ± 3.6	25.7 ± 2.9	0.21
Body mass index ≥30 kg/m^2 (%)	13.2	6.8	0.21
Alaninoaminotransferase (IU)	88.1 ± 73.8	76.5 ± 51.2	0.30
Platelets (10^3/mmc)	204.5 ± 83.1	212.2 ± 65.5	0.54
Cholesterol (mg/dl)	174.0 ± 34.0	163.6 ± 34.9	0.04
Triglycerides (mg/dl)	95.8 ± 47.0	77.9 ± 31.6	0.01
Blood glucose (mg/dl)	96.1 ± 27.0	93.7 ± 19.3	0.56
Insulin (μU/ml)	13.8 ± 9.4	11.8 ± 7.6	0.30
HOMA	3.39 ± 2.25	3.15 ± 1.78	0.49
Type 2 diabetes (%)	13.0	9.1	0.44
IL28B TT/TC/CC	16.8/54.0/29.2	22.7/54.6/22.7	0.48
PNPLA3 CC/CG/GG	54.3/38.2/7.5	59.1/29.5/11.4	0.41
HCVRNA (LOG)	5.9 ± 0.6	5.8 ± 0.6	0.45
Histology at biopsy			
Steatosis grade (%)			
Absent/mild-moderate/severe	54.8/28.8/16.4	47.7/31.8/20.5	0.63
Necroinflammatory activity (%)			
Severe	43.8	38.6	0.50
Fibrosis (%)			
Absent/mild-moderate/severe	30.9	25.0	0.40

yrs, years; IU, international units; HOMA, homoeostasis model assessment. Data are given as mean ± SD or as %.

Associations of gene variants with steatosis severity in G1 CHC patients

Patients carrying the *TM6SF2* rs58542926 variant had a similar distribution of steatosis severity as noncarriers. Percentages of patients with absent, mild-moderate and severe steatosis were 54.8%, 28.8% and 16.4%, respectively, in carriers compared to 47.7%, 31.8% and 20.5%, respectively, in noncarriers ($P = 0.63$, Fig. 1A). Prevalence and severity of steatosis were lower in patients carrying the *IL28B* C allele ($P = 0.005$, Fig. 1B) and elevated in those carrying the *PNPLA3* G allele ($P < 0.001$, Fig. 1C) compared to the corresponding noncarriers.

A multivariate ordinal regression analysis adjusted for age, gender, BMI and HOMA score showed that the aforementioned variants of *IL28B* (OR 0.69, 95% CI 0.55–0.86, $P = 0.001$) and *PNPLA3* (OR 1.84, 95% CI 1.46–2.83, $P < 0.001$), but not of *TM6SF2* (OR 1.48, 95% CI 0.82–2.69, $P = 0.19$), were independently associated with the severity of steatosis (Table 2). Severity of steatosis was also associated with age (OR 1.02, 95% CI 1.01–1.03, $P < 0.001$), BMI (OR 1.13, 95% CI 1.08–1.18, $P < 0.001$) and HOMA score (OR 1.08, 95% CI 1.01–1.16, $P = 0.01$). When T2DM was included in the model instead of HOMA, similar results were observed. The lack of association between the *TM6SF2* variant and steatosis severity, as well as the independent link between the *IL28B* and *PNPLA3* variants and steatosis severity, were further confirmed in subgroups of nondiabetic or nonobese patients (data not shown).

When we considered severe steatosis (≥30%) as a dichotomous variable, BMI (OR 1.08, 95% CI 1.02–1.14, $P = 0.007$), HOMA score (OR 1.13, 95% CI 1.02–1.25, $P = 0.01$) and the *PNPLA3* variant (OR 1.98, 95% CI 1.38–2.84, $P < 0.001$), but not the *TM6SF2* variant (OR 1.49, 95% CI 0.60–3.73, $P = 0.38$), were

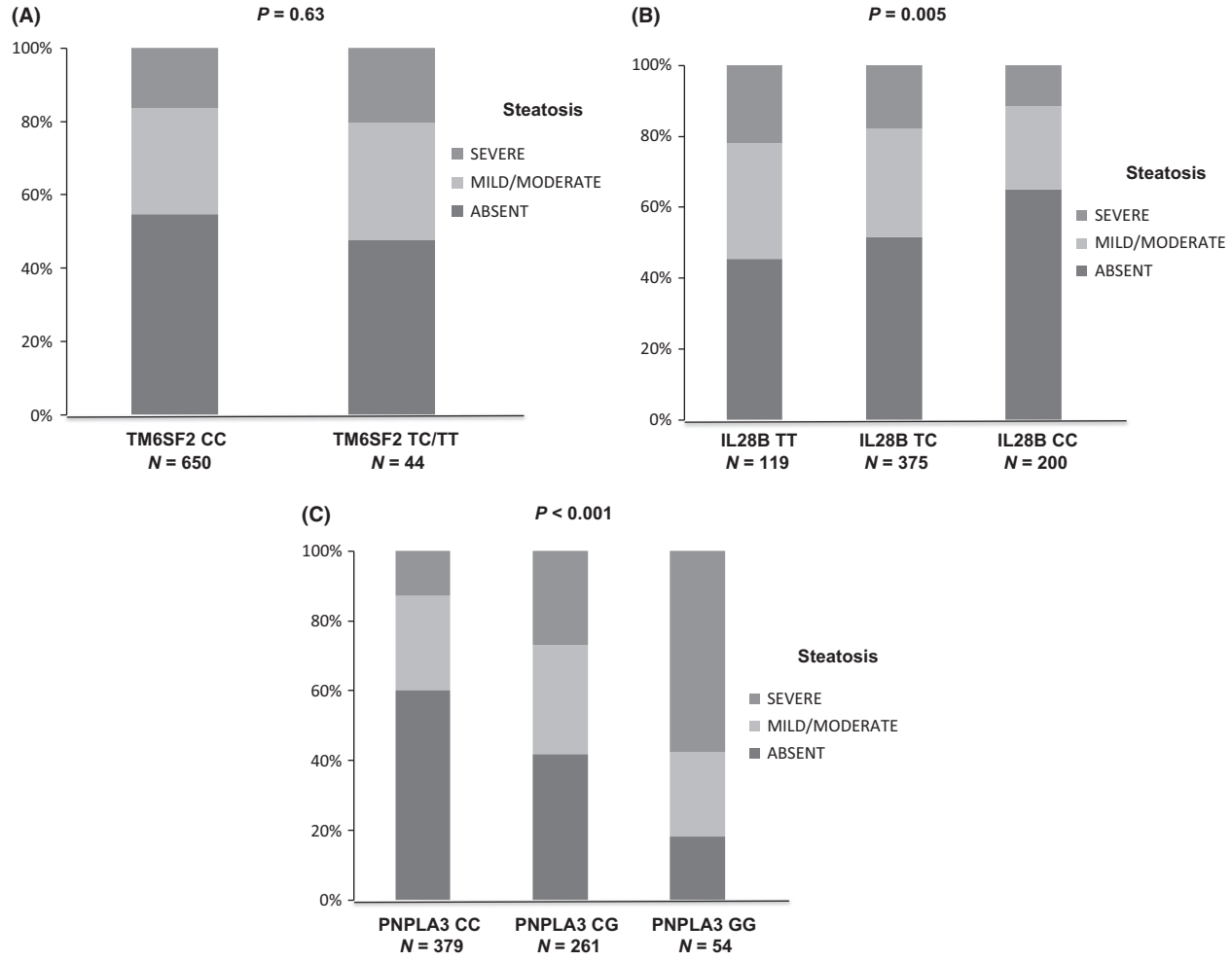

Fig. 1. Severity of steatosis according to *TM6SF2* (A), *IL28B* (B) and *PNPLA3* (C) genotypes.

Table 2. Factors associated with severity of steatosis in 694 patients with genotype 1 chronic hepatitis C

	Simple ordinal regression		Multiple ordinal regression	
Variable	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age (yrs)	1.02 (1.01–1.03)	<0.001	1.02 (1.01–1.03)	<0.001
Female gender	1.12 (0.84–1.50)	0.41	1.04 (0.76–1.41)	0.79
Body mass index (kg/m^2)	1.14 (1.09–1.19)	<0.001	1.13 (1.08–1.18)	<0.001
HOMA	1.16 (1.09–1.25)	<0.001	1.08 (1.01–1.16)	0.01
TM6SF2 CC/CT/TT	1.32 (0.74–2.34)	0.34	1.48 (0.82–2.69)	0.19
IL28B TT/TC/CC	0.66 (0.53–0.82)	<0.001	0.69 (0.55–0.86)	0.001
PNPLA3 CC/CG/GG	1.74 (1.38–2.17)	<0.001	1.84 (1.46–2.33)	<0.001

yrs, years; IU, international units; HOMA, homoeostasis model assessment. Data are given as mean ± SD or as %.

independent risk factors by multivariate logistic regression analysis. Similarly, steatosis as continuous variable was independently associated with older age ($P = 0.04$), higher BMI ($P < 0.001$), higher HOMA score ($P = 0.001$), lack of the *IL28B* C allele ($P = 0.01$) and presence of the *PNPLA3* G allele ($P < 0.001$), but not with presence of the *TM6SF2* T allele ($P = 0.07$), by multivariate linear regression analysis.

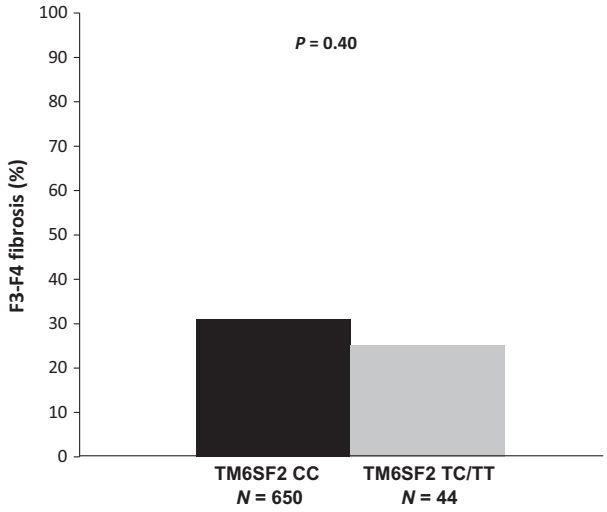

Fig. 2. Prevalence of F3-F4 fibrosis according to the *TM6SF2* genotype.

Association of gene variants and steatosis severity with fibrosis severity in CHC patients

The prevalence of F3-F4 fibrosis was similar in patients with the *TM6SF2* CC genotype compared to the CT genotype (30.9% vs. 25%, $P = 0.40$; Fig. 2). Simple logistic regression revealed that older age, higher HOMA score, severity of steatosis and severe necroinflammatory activity were associated with severe fibrosis ($P < 0.01$, Table 3). Multiple logistic regression confirmed that age (OR 1.04, 95% CI 1.02–1.06, $P < 0.001$), HOMA score (OR 1.23, 95% CI 1.12–1.35, $P < 0.001$), severity of steatosis (OR 1.36, 95% CI 1.06–1.75, $P = 0.01$) and severe necroinflammatory activity (OR 3.55, 95% CI 2.46–5.12, $P < 0.001$), but not the *TM6SF2* variant (OR 0.75, 95% CI 0.34–1.63, $P = 0.47$), the *IL28B* variant (OR 0.98, 95% CI 0.74–1.29, $P = 0.90$), or the *PNPLA3* variant (OR 0.99, 95% CI 0.74–1.32, $P = 0.96$), were independent predictors of severe fibrosis (Table 3). None of the gene variants remained associated with severe fibrosis when histological variables were removed from the model.

Discussion

We demonstrated that the *TM6SF2* rs58542926 variant does not affect the severity of liver damage in terms of steatosis or fibrosis, using a large cohort of Caucasian patients with G1 CHC and a low prevalence of obesity. Furthermore, we confirmed the key roles of *IL28B* and *PNPLA3* gene variants as steatosis modifiers.

Several lines of evidence in CHC have demonstrated that hepatic steatosis and some of its genetic determinants (e.g. *PNPLA3* and *IL28B* gene variants) affect the severity of liver damage and the liver-related prognosis of patients (3,15). Considering the recent evidence that *TM6SF2* rs58542926 promotes steatosis and liver damage in NAFLD (17–20), we examined its potential role in CHC. Our findings confirmed the low prevalence of the *TM6SF2* variant in a population with G1 CHC, as well as the association of this gene variant with lower TC and TG levels (17,19,20,26). We further confirmed the lack of an association between the *TM6SF2* genotype and IR, as was reported in an elegant study by Zhou and colleagues (27). Unexpectedly, we found no association between the *TM6SF2* variant and the presence or severity of steatosis in G1 CHC patients after adjusting for genetic and metabolic confounders. This result was further confirmed in subgroups of nondiabetic patients and nonobese patients, as well as by sorting steatosis at different thresholds.

The strength of our study lies in the large number of patients enrolled. To date, this study uses the largest cohort of G1 CHC patients to investigate the association between the *TM6SF2* variant and steatosis in a clinical setting. Our results are not inconsistent with

Table 3. Factors associated with severe fibrosis in 694 patients with genotype 1 chronic hepatitis C

	Simple logistic regression		Multiple logistic regression	
Variable	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age (yrs)	1.04 (1.03–1.06)	<0.001	1.04 (1.02–1.06)	<0.001
Female gender	0.76 (0.55–1.05)	0.10	0.70 (0.48–1.01)	0.06
Body mass index (kg/m^2)	1.00 (0.96–1.05)	0.76	0.94 (0.89–1.01)	0.06
HOMA	1.23 (1.13–1.34)	<0.001	1.23 (1.12–1.35)	<0.001
TM6SF2 CC/CT/TT	0.74 (0.36–1.50)	0.41	0.75 (0.34–1.63)	0.47
IL28B TT/TC/CC	0.94 (0.74–1.20)	0.64	0.98 (0.74–1.29)	0.90
PNPLA3 CC/CG/GG	1.02 (0.79–1.31)	0.86	0.99 (0.74–1.32)	0.96
Severity of steatosis	1.60 (1.30–1.98)	<0.001	1.36 (1.06–1.75)	0.01
Severe necroinflammatory activity	3.95 (2.81–5.57)	<0.001	3.55 (2.46–5.12)	<0.001

yrs, years; IU, international units; HOMA, homoeostasis model assessment. Data are given as mean ± SD or as %.

the exome-wide study by Kozlitina *et al.* (17). Although their study was population-based, we looked at the *TM6SF2* gene variant as a risk factor for steatosis in a population of CHC patients, who have a higher incidence of steatosis compared to the general population (28).

On the other hand, our data do not agree with data from two recent studies on CHC (21,22). Coppola and colleagues (21) found an association between steatosis and the *TM6SF2* variant in a small cohort (n = 148) of CHC patients with different HCV genotypes. Similarly, Milano and colleagues (22) identified a link between the *TM6SF2* variant and steatosis in 815 CHC patients, confirming this association in the subgroup of 451 G1 patients only. Several factors may account for the inconsistent results between studies, including the low number of patients evaluated in proportion to the low prevalence of the *TM6SF2* variant (21), the inclusion of patients with different CHC genotypes (21,22), as well as differences in histological methods (21,22), mean BMI (22) and the percentage of patients with moderate alcohol intake (22). Our study results confirm the modulation of steatosis and its severity by variants in the *PNPLA3* and *IL28B* genes in patients with CHC (8,9,11,12).

The lack of an association between the *TM6SF2* variant and steatosis in CHC may be explained by considering the mechanisms leading to steatosis in CHC, and the mechanisms by which the *TM6SF2* variant promotes liver fat accumulation. In patients with CHC, steatosis is a prevalent histological finding related to metabolic and viral factors, including induction of IR (especially for G1 patients) and impairment of lipid export from the liver (especially for, but not exclusive to, G3 patients) (28). In experimental models, silencing *TM6SF2* reduces the secretion of very low density lipoproteins and causes a predisposition to fatty liver development (17,26). Therefore, although the *TM6SF2* variant does not interfere with IR (27), both HCV infection and *TM6SF2* promote steatosis by affecting lipid export from the liver, although by different pathways. Accordingly, we might hypothesize that the steatogenic effect of the *TM6SF2* variant could be clinically less relevant in CHC patients.

We did not find any association between the *TM6SF2* variant and liver fibrosis, as was observed in NAFLD patients (18–20) and CHC patients (22). In NAFLD, the link between fibrosis and the *TM6SF2* variant is mediated by steatohepatitis. Thus, the lack of an effect of the *TM6SF2* variant on liver fat accumulation in our CHC cohort could explain the absence of a link with the severity of fibrosis. In our population, *PNPLA3* and *IL28B* gene variants were not directly associated with F3-F4 fibrosis, although their effects were mediated by steatosis induction. Further studies are necessary to establish how the HCV genotype and other clinical characteristics impact the effect of the *TM6SF2* variant on steatosis and liver damage in CHC.

From a clinical perspective, our data confirm the roles of the *PNPLA3* and *IL28B* gene variants, but not the *TM6SF2* variant, as major disease-modifying factors in CHC that promote histological steatosis, which contributes to the progression of liver disease. If confirmed in other studies performed in other geographical and clinical settings, this relationship would suggest that genotyping of *PNPLA3* and *IL28B*, but not *TM6SF2*, should be undertaken in CHC for risk stratification and personalized disease management, with more intensive follow-up of patients with the variant genotype, both before and after viral eradication.

The main limitation of this study lies in the potentially limited external validity of the results for different populations and settings. Thus, replicating our results is important. Lack of data on body fat distribution, serum levels of adipocytokines and other polymorphisms that could conceivably confound the data may have also affected the results. In conclusion, we found that in a large cohort of G1 CHC patients with a low prevalence of obesity, the *TM6SF2* rs58542926 variant does not affect the severity of liver damage in terms of steatosis or fibrosis. However, we did confirm the key roles of *IL28B* and *PNPLA3* gene variants as steatosis modifiers. These data, if further validated, could be useful in managing patients before and after therapy.

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