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Diverse impacts of the rs58542926 E167K variant in TM6SF2 on viral and metabolic liver disease phenotypes

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Abstract (N=264 words)

A genome-wide exome association study has identified the transmembrane 6 superfamily member 2 (*TM6SF2) rs58542926* variant encoding an E167K substitution as a genetic determinant of hepatic steatosis in nonalcoholic fatty liver disease (NAFLD). The role of this variant across a spectrum of liver diseases and pathologies and on serum lipids comparing viral hepatitis to NAFLD, and viral load in chronic viral hepatitis, and its intrahepatic molecular signature, have not been well characterized. We undertook detailed analyses in 3,260 subjects with viral and non-viral liver diseases and in healthy controls. Serum inflammatory markers and hepatic expression of *TM6SF2* and genes regulating lipid metabolism were assessed in a subset with chronic hepatitis C (CHC). The *rs58542926* T allele was more prevalent in 502 NAFLD patients than controls (p=0.02), but not different in cohorts with CHC (n = 2,023) and CHB (n = 507). The T allele was associated with alterations in serum lipids and hepatic steatosis in all diseases, and with reduced hepatic *TM6SF2* and *MTTP* expression expression. Interestingly, the substitution was associated with reduced CHC viral load, but increased HBV-DNA. The *rs58542926* T allele had no effect on inflammation, impacted on ≥F2 fibrosis in CHC and NAFLD assessed cross-sectionally (OR: 1.39 (95% CI = 1.04-1.87 and OR: 1.62, 95% CI: 1.03-2.52, respectively, p<0.03 for both), but had no effect on fibrosis progression in 1174 patients with CHC and a known duration of infection. We conclude that the *TM6SF2* E167K substitution promotes steatosis and lipid abnormalities in part by altering *TM6SF2* and *MTTP* expression and differentially impacts CHC and CHB viral load, while effects on fibrosis are marginal.

INTRODUCTION

Ectopic lipid accumulation associated with the increasing worldwide prevalence of obesity ([1]) is linked with tissue meta-inflammation ([2]), resulting in organ dysfunction, excess morbidity and mortality ([3]). In this context, hepatic rather than visceral fat deposition is more tightly linked with the metabolic complications of obesity ([4]) and it is therefore not surprising that steatosis is now the most commonly observed histological abnormality across a spectrum of liver diseases ([5]). The heritability of steatosis is therefore of considerable interest, with a landmark genome-wide association study (GWAS) ([6]) and subsequent reports ([7]) demonstrating unequivocal evidence that a non-synonymous single-nucleotide polymorphism (SNP) in patatin-like phospholipase domain containing 3 (*PNPLA3) rs738409* is associated not only with steatosis severity, but with the extent of fibrosis in NAFLD ([8]). More recently, in 7176 individuals of European ancestry, a GWAS meta-analysis in NAFLD using 2.4 million SNPs imputed to HapMap, identified additional common variants at 5 loci containing multiple genes (NCAN/TM6SF2/CILP2/PBX4) that impacts steatosis ([7]). A subsequent genome-wide exome association study with functional analysis ([9]) identified an *rs58542926 C>T* variant in the transmembrane 6 superfamily member 2 (*TM6SF2*) gene, which encodes an amino acidic substitution (E167K) as the causative variant for the GWAS signal. The impact of *TM6SF2 rs58542926* as a determinant of liver fat in NAFLD is well-established ([10,11,12]). While there is evidence suggesting a role for this variant in NAFLD-associated liver fibrosis, this finding is not unequivocal ([10,11,12,13,14]), and is debatable in the case of CHC ([15,16]). There is no data on the role of *rs58542926* in chronic hepatitis B (CHB).

With regard to the metabolic consequences of harboring the *rs58542926* T allele, an association with lower levels of serum total cholesterol, LDL-cholesterol (LDL-C) and triglycerides (TG), and protection from atherosclerosis and cardiovascular events in NAFLD

([9,11,17]) has been reported. This cardiovascular risk mitigation is likely a consequence of data (from mouse models and cell lines), suggesting that TM6SF2 modulates hepatic lipoprotein export ([9,18]). However, corroborating human data is absent, but required, given the limitations of the cell lines used in published reports ([15]). VLDL secretion is nearly absent in Huh7 cells ([19]) and HepG2 cells secrete relatively dense, lipid poor apolipoprotein B (apoB)-containing particles, unlike the buoyant VLDL particles secreted *in vivo* ([20]). The impact of *TM6SF2 rs58542926* polymorphisms on serum lipoproteins in patients with viral hepatitis is unknown.

A third unexplored but plausible consequence of TM6SF2 action is influences on viral load in chronic viral hepatitis. Both chronic hepatitis C and B (CHC and CHB) are associated with abnormalities in lipid metabolism ([21,22,23]), lipids play important roles in various aspects of the life cycle of the viruses ([24,25]), and we have shown that a variable fraction of HCV in serum is associated with triglyceride-rich lipoproteins (TRLs) called lipoviral particles that exhibit increased infectivity ([26]). Thus, it could be hypothesized that gene variants that modulate hepatic lipid metabolism and TG export could impact on viral load.

Based on these considerations, the aims of the present study were to clarify the influence of the *TM6SF2 rs58542926* variant on the metabolic and histological features of liver diseases, comparing viral hepatitis to NAFLD, to explore effects on viral load and thirdly to investigate the impact of carriage of the T allele on the expression of hepatic *TM6SF2* and genes modulating liver lipid accumulation.

Methods

Patient cohort

The study comprised 3,260 consecutive subjects from the International Liver Disease Genetics Consortium (ILDGC) database. Details of the cohort and inclusion criteria have been reported ([27]). Briefly, for those with CHC, patients who had a liver biopsy with scoring for fibrosis stage and disease activity before anti-viral treatment were included. Patients were excluded if they had evidence of other liver diseases by standard tests. In addition to the 2,023 with CHC, the cohort comprised 507 patients with CHB, 502 with NAFLD and 228 healthy controls. Only one of the patients with CHC was included in a recent report on TM6SF2 ([15]). The healthy Caucasian European control group was enrolled from Westmead hospital, Sydney, and reported no history of chronic liver disease. Details of the selection criteria for control subjects are presented in supplementary methods.

Ethics approval was obtained from the Human Research Ethics Committees of the Sydney West Local Health District and the University of Sydney. All other sites had ethics approval from their respective ethics committees. Written informed consent for genetic testing was obtained from all participants.

Clinical and laboratory assessment

A detailed description of the baseline data collected at the time of liver biopsy and methods to estimate the duration of infection is provided in supplementary methods. Data included demographic parameters, alcohol intake, and routine laboratory variables including metabolic parameters.

Genotyping

Genotyping for *TM6SF2 rs58542926* and *PNPLA3 rs738409* was contracted to the Australian Genome Research Facility (AGRF; QLD, Australia). Samples were genotyped using the Sequenom MassARRAY system and iPLEX Gold chemistry. Further genotyping for both SNPs (n=1150) was undertaken using the TaqMan SNP genotyping allelic discrimination method (Applied Biosystems, Foster City, CA, USA). All genotyping was blinded to clinical variables.

Liver Histopathology

Liver histopathology was scored according to METAVIR ([28]). Fibrosis was staged from F0 (no fibrosis) to F4 (cirrhosis). Necroinflammation (A) was graded as A0 (absent), A1 (mild), A2 (moderate), or A3 (severe). For NAFLD, the Kleiner classification was used ([29]).According to the classification of Bedossa et al., ([30]) histological features of steatohepatitis in CHC was also assessed in a subset of 112 patients infected with genotype 1. The inter-observer agreement between pathologists was studied previously and was good (κ = 77.5) for METAVIR staging using κ statistics ([31]). Histopathology methods are detailed in the supplementary methods.

Determination of inflammatory markers and mRNA levels of genes regulating lipid metabolism

Ninety well characterized patients with CHC underwent liver biopsy at Westmead Hospital, their details have been described previously ([32]). We assessed the relationship between *rs58542926* genotype and the expression of *TM6SF2,* acyl-coenzyme A oxidase 1 (*ACOX1*) and microsomal triglyceride transfer protein (*MTTP*). The expression of sterol regulatory element binding protein-1c (*SREBP-1c*) and fatty acid synthase (*FAS*) was measured previously ([32]). The detailed methods have been described previously ([32]) and summarized in the supplementary

methods. Serum TNF-α, soluble tumor necrosis factor receptor 2 (sTNFR2) and IL-6 in these patients were measured by sandwich enzyme-linked immunosorbent assay (ELISA).

Statistical Analysis

Statistical methods are detailed in the supplementary methods. All tests were two-tailed and p values <0.05 were considered significant.

Results

Patient characteristics

The study comprised 3,260 consecutive subjects including 2,023 with CHC, 507 with CHB, 502 with NAFLD and 228 healthy controls. The clinical, anthropometric, biochemical and metabolic characteristic of the patients in the CHC cohort are presented in **supplementary table 1**. The genotype distribution of *TM6SF2 rs58542926* and *PNPLA3 rs738409* was in Hardy-Weinberg equilibrium **supplementary table 2**.

Association between the *TM6SF2* variant and viral, clinical and metabolic characteristics in CHC

The minor allele frequency (MAF) of *TM6SF2 rs58542926* was 0.06, similar to that of the 228 Caucasian self-reported 'healthy' controls (MAF 0.07) (p=0.7 for trend) **supplementary table 1**. There was no significant difference in the *rs58542926* genotype distribution according to patient country of origin (Australia, UK, Germany and Italy) (p=0.1 for trend).

Patients with the *TM6SF2 rs58542926 TT* genotype had significantly lower HCV viral load compared to subjects with CT or CC (p=0.007), after Bonferroni correction for multiple comparisons (**Table 1**). In another analysis, mean HCV viral load was lower in subjects with CT/TT compared to those with CC (5.74±0.73 vs. 5.59±0.0.76; p=0.02). To adjust for the effect of confounding factors, *TM6SF2 rs58542926 TT* genotype still associated inversely with HCV-RNA levels in a multivariate model adjusting for age, gender, HCV-genotype, BMI, HOMA-IR, lipid profile and *PNPAL3* genotype (adjusted estimate, -0.144; SE, 0.082; p=0.001). The distribution of *rs58542926* genotype was not different according to HCV genotype.

As regards the metabolic profile, subjects with the *TT* genotype had significantly lower total cholesterol, LDL-C and TG, while paradoxically having significantly higher BMI and a

trend to higher HOMA-IR. The median of ALT, AST, GGT and ALP levels, and platelet counts were not significantly different between *rs58542926* genotypes (**Table 1**). Similar results were observed when comparing CT/TT vs. CC (data not shown). For the purposes of comparison, the association of *PNPLA3 rs738409* with the same variables was investigated. Apart from the fact that subjects with *rs738409* GG genotype were significantly younger and had lower fasting blood glucose level, compared to subjects with CG and CC, no other significant associations were observed (**Supplementary Table 3**).

Association between the *TM6SF2* variant and histological features in the cohort with CHC

We next assessed in the CHC cohort, the association between *TM6SF2 rs58542926* and histological features including steatosis, features of steatohepatitis (in 112 subjects), inflammation and fibrosis. The distribution of TM6SF2 *rs58542926* genotypes according to histological features (steatosis grade, inflammation, and fibrosis) are depicted in **Supplementary Figure 1.** The *rs58542926* T allele had a modest association with the degree of steatosis in a multivariate model adjusting for age, gender, HCV-genotype, BMI, HOMA-IR and *PNPAL3* genotype (adjusted estimate, 0.073; SE, 0.002; p=0.01) **(Supplementary Table 4)**. When stratifying the cohort according to HCV genotype (genotype 3 vs. non-3) based on the direct effect of genotype 3 on steatosis ([33]), the *rs58542926* was associated with steatosis only in non-HCV-genotype 3 patients (adjusted estimate, 0.112, SE, 0.006; p=0.04). In contrast, *PNPLA3* rs738409 genotype was the strongest predictor of steatosis in both non-genotype 3 (adjusted estimate, 0.199, SE, 0.14; p=0.005) and in genotype 3 patients (adjusted estimate, 0.127, SE, 0.077; p=0.03) **(Supplementary Table 4)**.

We explored the association of *rs58542926* with the severity of steatosis by subdividing the cohort according to absent/mild steatosis (S0-S1) compared to moderate/severe steatosis (S2-

S3). In this analysis, *rs58542926* was associated with the severity of steatosis (OR: 1.14, 95%CI: 1.02-1.27, p=0.01), independent of *PNPLA3 rs738409* (OR: 4.3, 95%CI: 1.94-9.52, p <0.0001) and other clinical variables (**Table 2**).

We then assessed the role of the TM6SF2 variant on hepatic inflammation. In multivariate analysis, neither *TM6SF2 rs58542926*, nor *PNPLA3 rs738409* demonstrated any association with inflammation (**Supplementary Table 5**). No association was also observed when the cohort was dichotomized into absent/mild (A0-A1) and moderate/severe (A2-A3) inflammation. In sub-analysis of 112 subjects with genotype 1 CHC, we investigated the possible association between *TM6SF2 rs58542926* and features of 'steatohepatitis' in CHC according to the Bedossa classification ([30]) and no association was observed (data not shown). Furthermore, no difference was noted in serum inflammatory markers (TNF-α, sTNFR2 and IL-6) or liver enzymes (ALT or AST; as indices of liver injury) according to *rs58542926* genotype.

Lastly, in the cross sectional analysis, we examined the impact of *TM6SF2 rs58542926* genotype on fibrosis. The *rs58542926* SNP was marginally associated with fibrosis stage in univariate analysis, but not in multivariate linear regression analysis controlling for age, gender, steatosis, HOMA-IR, BMI, HCV genotype, alcohol intake and *PNPLA3* genotype (adjusted estimate, 0.071, SE, 0.003; p=0.06) (**Supplementary Table 5**). The adjusted OR for having significant fibrosis (≥F2) was 1.39 (95% CI: 1.04-1.87, p=0.02) for the T allele, and with cirrhosis (>F4) was 1.82 (95% CI: 1.01-3.28, p=0.04) (**Table 2**).

Association between the *TM6SF2* variant and fibrosis progression

Given the inherent biases in cross sectional analyses, the lack of impact of *TM6SF2 rs58542926* genotype on hepatic inflammation, the principal driver of liver fibrosis, and the modest impact on fibrosis stage (absent in multivariate linear regression and present by

multivariate logistic regression), we undertook further analysis in 1,174 patients with CHC and a known duration of infection, allowing us to assess the relationship with fibrosis progression. The baseline characteristics of the cohort were similar among subjects included and not included in the fibrosis progression sub-analysis (**Supplementary Table 6).** The OR of *rs58542926* for having fast FPR was 1.31 (95% CI: 1.08-2.97, p=0.03), while after adjustment, the T allele was not independently associated with FPR (p= 0.5) (**Supplementary Table 7).**

For further confirmation, we assessed fibrosis progression using Cox-proportional hazards to address the concern that FPR may not be constant over time ([34]). *rs58542926* was not associated with an increased hazard of progression to significant fibrosis (≥ F2) in univariate or multivariate models (hazards ratio: 1.43, 95% CI: 0.64-3.21, p=0.3), including when adopting other genetic models. In contrast, *rs738409* in *PNPLA3* showed a trend for significance (hazards ratio: 1.34, 95% CI: 0.97-1.84, p=0.06), but again not in a multivariate model (**Figure 1**). In another model, *rs58542926* was not associated with an increased hazard of progression to cirrhosis (F4) (hazards ratio: 1.16, 95% CI: 0.16-8.39, p=0.8), **supplementary figure 2**.

There was no evidence of interaction between *rs58542926* and *rs738409* on any histological features (steatosis, inflammation and fibrosis) (**data not shown**).

In sum, the *TM6SF2 rs58542926* T allele was associated with a modest risk of hepatic steatosis in CHC, being more profound in subjects with non-genotype 3 infection. There was no effect of the T allele on inflammation, while an effect on fibrosis stage was at best, modest, with no effect on fibrosis progression. The effect of the *TM6SF2 rs58542926* T allele was independent of *PNPLA3* 738409.

Relationships between *rs58542926* genotype and hepatic gene expression

There was a significant relationship between carriage of the *rs58542926 T* allele and lower *TM6SF2* mRNA expression (p=0.001, **Figure 2 B**). Expression of *SREBP1c*, *FAS* and *ACOX1* mRNA, as markers of lipid synthesis and oxidation, were not significantly different according to *rs58542926* genotype. As expected however, serum TG was significantly lower in subjects with the *rs58542926* CT/TT than CC genotype (**Figure 2 C)**. The expression of *MTTP*, a key protein involved in TG packaging and export was significantly lower in liver from subjects with the T allele (**Figure 2**C). These results suggest that in CHC, carriers of the T allele for *rs58542926* have reduced *TM6SF2* and *MTTP* expression, concomitant with reduced serum TG levels.

Other chronic liver diseases

Chronic hepatitis B

The characteristics of 507 Chinese patients with CHB are summarized in **Supplementary Table 8**. The *TM6SF2 rs58542926* MAF of 0.07 was similar to that observed in Caucasian populations, and in other Han Chinese population cohorts ([13]) (**Supplementary Table 1)**. The distribution of TM6SF2 rs58542926 genotypes according to histological features (steatosis grade, inflammation, and fibrosis) are depicted in **Supplementary Figure 3**. The *rs58542926* T allele was associated with steatosis (adjusted estimate, 0.094; SE, 0.003; p=0.03), but, in particular, with presence of any steatosis (S0 versus S $\geq$1) (OR=2.57, 95%CI: 1.36-4.86, P = 0.003) rather than with severe steatosis (S0-S1 versus S2-S3) (OR=1.49, 95%CI: 0.83-2.67, P = 0.1) **(Table 3).** No association was observed between *rs58542926* genotype and inflammation or fibrosis (**Supplementary Table 9**). Notably, only classic risk factors such as

age and gender demonstrated an independent association with fibrosis (**Supplementary Table 10**), and *PNPLA3 rs738409* showed no association with either inflammation or fibrosis.

Again, as noted in other liver diseases cohorts, subjects with the T allele had lower serum TG and LDL-C, but this was significant only for the latter (p=0.008). Interestingly, in contrast to what was observed in CHC, patients with CHB had higher HBV-DNA levels (p=0.04) (**Supplementary Table 11)**. The association with HBV-DNA levels remained significant after adjusting for age, gender, BMI, presence of diabetes, lipid profile and *PNPAL3* genotype (adjusted estimate, 0.103; SE, 0.036; p=0.03). For *PNPLA3 rs738409*, apart from higher serum HDL-C in subjects with the GG genotype, no other significant associations were demonstrated (**Supplementary Table 12)**.

NAFLD

The characteristics of 502 Caucasian patients with NAFLD are summarized in **Supplementary Table 13.** The MAF for *rs58542926* in the NAFLD cohort was 0.12 with an increase in T allele frequency compared to healthy controls (X^2 for trend, P=0.01; **Supplementary Table 1**). The distribution of TM6SF2 rs58542926 genotypes according to histological features (steatosis grade, inflammation, and fibrosis) are depicted in **Supplementary Figure 4**. *rs58542926* was associated with the risk of fatty liver (adjusted estimate, 0.111; SE, 0.097; p=0.02); this was independent of age, gender, BMI, presence of diabetes, hypertension, and *PNPLA3 rs738409* genotype (**Supplementary Table 14**).

rs58542926 demonstrated an independent association with stage of fibrosis (adjusted estimate, 0.189; SE, 0.117; p=0.003) and with risk of significant fibrosis (≥ F2) in multiple logistic regression analysis (OR:1.62, 95% CI: 1.03.-2.52, p=0.03) (**Table 4**). Nevertheless, analysis of the relationship between *rs58542926* and steatohepatitis did not demonstrate a

significant association (p=0.6) (**Supplementary Table 14**). Likewise, we observed a lack of association between the gene variant and the NAS score (**data not shown**). There was no interaction between *PNPLA3 rs738409* and *TM6SF2 rs58542926* variants on any histological features (steatosis, steatohepatitis or fibrosis) in NAFLD.

As expected, subjects with the *rs58542926* TT genotype had lower serum TG and ALP levels, while no association was demonstrated with either serum ALT or AST values or with other metabolic parameters (**Supplementary Table 15**). As a control, the *rs738409 PNPLA3 GG* genotype demonstrated significantly higher ALT, AST and GGT (**Supplementary Table 16**), consistent with its independent association with steatohepatitis (adjusted estimate, 0.103; SE, 0.048; p=0.03), but no association was observed with the serum lipid profile. In a joint analysis of all cohorts (HCV, HBV, NAFLD), rs58542926 genotype showed no association with ALT, AST or GGT.

Discussion

We clarified the influence of the *TM6SF2 rs58542926* variant on the metabolic and histological features of viral hepatitis as compared to that in NAFLD. The most noteworthy finding was that the E167K substitution had a consistent effect on the serum metabolic profile and the predisposition to hepatic steatosis, rather than to hepatic inflammation and fibrosis across all three diseases. This effect was independent and not additive to the influence of the *PNPLA3 rs738409* C/G (I148M) polymorphism. Consistent with a crucial role for lipids in hepatitis virus replication, a novel observation was that the *TM6SF2* T allele has significant and opposite associations with HCV-RNA and HBV-DNA levels. Mechanistically, we show in a subset of patients, that alteration in *MTTP* expression in the liver of carriers with the T allele, might contribute to the hepatic and serum metabolic abnormalities observed.

Across all analyses and disease cohorts, the *TM6SF2* T allele conferred a modest risk for hepatic steatosis with estimates varying from (0.111, 0.073 and 0.094) in NAFLD, CHC and CHB, respectively, which is less than the risk for PNPLA3 *rs738409* (0.168, 0.159 and 0.119 respectively), and for established risk factors such as T2DM, BMI and HCV genotype 3. Likewise, carriage of the T allele was associated with lower serum lipoproteins in NAFLD, and as we demonstrate for the first time, in chronic viral hepatitis. To gain insights on the function of *TM6SF2* in patients with liver disease, we investigated the correlation of *rs58542926* genotype with hepatic expression of *TM6SF2*. The *rs58542926* T allele was associated with lower hepatic *TM6SF2* gene expression, a finding consistent with that observed by gene expression and eQTL analysis of incidental liver biopsies from 206 patients undergoing aortic valve surgery ([18]). In that study, patients were segregated according to *rs10401969* rather than the *rs58542926* genotype. However, *rs10401969* is in strong linkage disequilibrium (LD) with the non synonymous variant *rs58542926 (r2* > 0.99) identified by the genome-wide exome association

study ([18]). This is also consistent with another recent study demonstrating that the rs58542926 T allele is associated with decreased gene and protein expression in liver of NAFLD patients ([12]).

Of interest, in the CHC sub-cohort, we show that *MTTP* expression, required for VLDL assembly and maturation was reduced with carriage of the T allele, while the expression of genes involved in lipid synthesis and oxidation (*SREBP1c*, *FAS* and *Acox1* mRNA) were unaffected.

This human data is consistent with, and adds to recent observations from *Tm6sf2* overexpression and knockdown in mice and *in vitro* studies demonstrating that TM6SF2 is a key modulator of TG-rich lipoproteins and apolipoprotein B (apoB) ([9,18]). In humans, TG is delivered to adipose tissues by intestinally-derived chylomicrons and by hepatic VLDL synthesis ([35]). Notably, both liver and intestine express the highest levels of *TM6SF2* ([9,18]). *MTTP* is absolutely required for VLDL assembly and maturation ([36]), catalyzing the transport of triglyceride, cholesteryl ester and phospholipids to the nascent apoB molecule, that is required for the assembly and secretion of VLDL and low-density lipoproteins (LDLs) ([37]). *MTTP* is found in abundance on the luminal side of the ER and in the liver, intestine, and heart ([38]), and thus it is of interest that a recent study using confocal microscopy observed localization of green fluorescent protein–tagged TM6SF2 to the ER of 2 human hepatoma cell lines (Huh7 and HepG2) ([18]). In that study, knockdown of *TM6SF2* in both cell lines had no effect on the expression of *APOB* or *MTTP*, however whether *MTTP* expression is altered based on TM6SF2 genotype was not tested.

Of particular interest is the significant and opposite association between the *TM6SF2* variant and HCV and HBV viral load, with the T allele associated with lower HCV-RNA levels but higher HBV-DNA. The association remained significant (p=0.001 with CHC and p=0.03 with CHB) after controlling for the main confounders. Although our analyses do not allow for a

mechanistic explanation, it is consistent with previous reports from population based studies and experimental models indicating that HCV-RNA levels correlate positively, while HBV-DNA levels correlate inversely with serum TG ([21-23]). From a teleological standpoint, the question remains how the E167K substitution in *TM6SF2 rs58542926* might favor HBV replication and inhibit HCV. Previous studies have shown that blocking VLDL secretion by inhibiting MTTP with naringenin reduces the export of HCV ([39]), while overexpression of seipin, a protein implicated in the maturation and fusion of lipid droplets (LDs) in hepatoma cells subsequently infected with HCV causes a significant decrease in virion export, accompanied by the appearance of large LDs (experimental steatosis) ([40]). Thus, our data if confirmed suggests that the functional consequence of the E167K substitution in *TM6SF2* by blocking VLDL export might also restrict HCV export and hence viral load.

Although the interaction of the HBV life cycle with lipid metabolism is not well characterized, it has been suggested that lipids play a vital role in HBV replication ([41]). While acute hepatitis B is associated with transient hypertriglyceridemia ([42]), CHB is associated with reduced serum TG and total cholesterol ([43]). Several studies suggest that the HBV X protein (HBx) induces lipid accumulation in HepG2–HBx stable cells and in HBx-transgenic mice ([44,45]) by activation of lipogenic and fatty acid synthesis genes such as *SREBP1* and *peroxisome proliferator-activated receptor γ* (*PPARγ)*, and inhibiting the secretion of apoB. Likewise, in HepG2–HBV-stable cells, virus replication upregulates adipogenic gene expression (*CCAAT/enhancer binding protein α* (*C/EBPα*), *PPARγ*, *adiponectin*, *liver X receptor α* (*LXRα*), *SREBP1c*, and *Fas*). Further, adiponectin and the PPARγ agonist rosiglitazone stimulate viral replication ([46]). Thus, promotion of lipid synthesis, and blockage of lipid secretion are important pathways in HBV infection suggesting that hepatic steatosis induced by the E167K substitution could promote HBV replication. However, this hypothesis needs further investigation.

A recent study has suggested that the prevalence of the *rs58542926* T allele is reduced in European Caucasians with CHC ([15]), a finding we have been unable to replicate in the total cohort with CHC or in the different European and Australian centers, or in a the cohort of Asians with chronic hepatitis B. The reason for this discrepancy is not clear. Of relevance, whether *rs58542926* is involved in HCV or HBV spontaneous clearance requires further investigation. The TM6SF2 rs58542926 MAF of 0.07 in our Chinese patients with CHB was similar to that observed in Caucasian populations, and in other Han Chinese population cohorts ([13]).

In our three cohorts, we were able to undertake robust analysis to assess the role of the E167K substitution on the more controversial aspects of liver histology, namely inflammation and fibrosis. The *TM6SF2 rs58542926* variant had no detectable effect on inflammation in all three chronic liver diseases, whether by histological grade, liver enzymes (ALT or AST) or in a subset with CHC, the levels of serum inflammatory markers (TNF-α, IL-6 and sTNFR2). Thus, clearly, if the E167K substitution in *TM6SF2 rs58542926* contributes to liver fibrosis, as has been suggested ([10,15]) it is likely mediated by a process other than hepatic necroinflammation.

To confirm or refute a role for the E167K substitution in *TM6SF2* on liver fibrosis, the cohort of nearly 1200 patients with CHC and a known duration of infection had us uniquely positioned to assess fibrosis progression, compared to all previous reports. In this analysis, *rs58542926* genotype had no effect on fibrosis progression assessed using either stage-constant fibrosis progression rates or Cox regression to model the time taken for significant fibrosis (≥ F2) or cirrhosis (≥ F4) to develop. Likewise, in cross sectional analyses of the total cohort with CHC (n=2023), the *rs58542926* T allele was not associated with fibrosis stage in adjusted multivariate linear regression analysis, while it only showed an association with ≥F2 fibrosis (OR: 1.39) and with cirrhosis (OR: 1.82) by logistic regression, consistent with a recent report ([15]). In CHB, likewise, we demonstrated no significant association of the *rs58542926* variant

with fibrosis stage, significant fibrosis (F2-F4), severe fibrosis (F3-F4) or with cirrhosis. In cross sectional analysis of the NAFLD cohort, as with previous reports ([10,11]), we confirmed an association of the *rs58542926* variant with more severe fibrosis stage and with significant fibrosis. The adjusted estimate and OR for *rs58542926* (β=0.136 and OR=1.62, respectively) was lower than these values for either *PNPLA3 rs738409* (β=0.1819 and OR=1.98, respectively) or classic clinical risk variables such as age, diabetes and hypertension.

Thus, in sum, our analyses indicates that the E167K substitution in *TM6SF2 rs58542926* has marginal if any effect on fibrosis in chronic viral hepatitis, and no effect on inflammation or fibrosis progression. In NAFLD, perhaps because steatosis occurs in the context of systemic metabolic dysregulation and oxidant stress from intra-hepatic toxic lipid species such as unesterified free fatty acids and ceramides ([47]), the *TM6SF2 rs58542926* variant more consistently amplifies and contributes to fibrosis. It should be noted, that the present analysis is unable to dissect whether the effect of the *TM6SF2* variant on fibrosis is direct or indirect through its association with metabolic derangements.

Unfortunately, data on viral clearance was not available in order to investigate the clinical relevance of our observations regarding *TM6SF2 rs58542926* with HCV and HBV viral load. Further, it should be acknowledged that population stratification was not assessed in detail. However, all participants with CHC and NAFLD were of Caucasian ancestry and all CHB patients were Chinese. It should be noted that population structure is more critical when studying rare variants ([48]), while *rs58542926* does not fit into this category (MAF=0.07), and we did not observe any difference in MAF between the different centres.

In conclusion, this is the first study to compare the diverse impacts of the rs58542926 E167K variant in TM6SF2 on the metabolic and histological features of metabolic and viral liver

disease. We demonstrate that the E167K substitution in TM6SF2 is associated with altered serum lipoproteins and susceptibility to hepatic steatosis in viral and non viral chronic liver disease, and with reduced hepatic MTTP expression in the HCV cohort. Though, the TM6SF2 rs58542926 minor allele influenced fibrosis in NAFLD, it had no discernable effects on liver inflammation, and perhaps only a minor influence on hepatic fibrosis, in chronic viral hepatitis. Importantly, the variant is associated with viral load, in opposing directions in CHC as compared to CHB, likely modulated by the differential effects of hepatic lipid metabolism and export, on the HBV and HCV virus life cycle.

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Figures legends:

Figure 1: Univariate Cox regression analysis of *TM6SF2 rs58542926* and *PNPLA3 rs738409* genotype on the cumulative probability of progression to moderate/severe (≥F2) fibrosis in 1174 patients with a known duration of HCV infection. Similar results was observed in multivariate Cox regression analysis after adjusting for covariates (age, gender, BMI, duration of the infection, HCV genotype, inflammation progression, and basal ALT, AST, GGT, platelets, bilirubin and alkaline phosphatase). Data of *TM6SF2 rs58542926* and *PNPLA3 rs738409* genotype are shown in dominant and additive models, respectively. No difference was observed in other genetic models.

Figure 2: A) Relationship between *rs58542926* genotype and serum triglyceride level in 1059 patients with CHC. In human liver samples from patients with CHC: B) Relationship between *rs58542926* genotype and *TM6SF2* mRNA levels C) Relationship between *rs58542926* genotype and the expression of sterol regulatory element binding protein-1c (*SREBP-1c*), fatty acid synthase (*FAS*), acyl-coenzyme A oxidase 1 (*ACOX1*) and microsomal triglyceride transfer protein (MTTP).

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