Research Article

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Impaired hepatic lipid synthesis from polyunsaturated fatty acids in *TM6SF2* E167K variant carriers with NAFLD

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Background: Carriers of the *transmembrane 6 superfamily member 2 E167K gene variant* (TM6SF2$^{EK/KK}$) have decreased expression of the *TM6SF2* gene and increased risk of NAFLD and NASH. Unlike common 'obese/metabolic' NAFLD, these subjects lack hypertriglyceridemia and have lower risk of cardiovascular disease. In animals, phosphatidylcholine (PC) deficiency results in a similar phenotype. PCs surround the core of VLDL consisting of triglycerides (TGs) and cholesteryl-esters (CEs). We determined the effect of the *TM6SF2* E167K on these lipids in the human liver and serum and on hepatic gene expression and studied the effect of *TM6SF2* knockdown on hepatocyte handling of these lipids.

Methods: Liver biopsies were taken from subjects characterized with respect to the TM6SF2 genotype, serum and liver lipidome, gene expression and histology. *In vitro*, after *TM6SF2* knockdown in HuH-7 cells, we compared incorporation of different fatty acids into TGs, CEs, and PCs.

Results: The TM6SF2$^{EK/KK}$ and TM6SF2^{EE} groups had similar age, gender, BMI and HOMA-IR. Liver TGs and CEs were higher and liver PCs lower in the TM6SF2$^{EK/KK}$ than the TM6SF2^{EE} group (p <0.05). Polyunsaturated fatty acids (PUFA) were deficient in liver and serum TGs and liver PCs but hepatic free fatty acids were relatively enriched in PUFA (p <0.05). Incorporation of PUFA into TGs and PCs in *TM6SF2* knockdown hepatocytes was decreased (p <0.05). Hepatic expression of *TM6SF2* was decreased in variant carriers, and was co-expressed with genes regulated by PUFAs.

Conclusions: Hepatic lipid synthesis from PUFAs is impaired and could contribute to deficiency in PCs and increased intrahepatic TG in *TM6SF2* E167K variant carriers.

Introduction

Common non-alcoholic fatty liver disease (NAFLD) is associated with features of the metabolic syndrome, such as hypertriglyceridemia, and increased risk of cardiovascular disease (CVD).[1] In 2014, a non-synonymous adenine-to-guanine substitution (rs58542926) replacing glutamate at residue 167 with lysine (E167K) in the transmembrane 6 superfamily member 2 (TM6SF2) protein was shown to increase liver fat content[2,3] and the risk of liver fibrosis.[4] In these and several subsequent studies, variant allele carriers (*TM6SF2*$^{EK/KK}$) were found to be neither more obese nor more insulin-resistant than non-carriers (*TM6SF2*^{EE}).[2,5–12] In contrast to most cases with 'obese/metabolic' NAFLD, *TM6SF2* variant allele carriers have normal[5,6,10,11] or decreased[2,8–10,12] plasma TG concentrations and a reduced risk of CVD.[12,13]

The exact function of the *TM6SF2* variant is unknown. In hepatocytes, *TM6SF2* minor allele carriage is associated with decreased TM6SF2 protein expression.[2] *TM6SF2* siRNA inhibition in hepatocytes increases cellular TG concentrations and reduces TG secretion, while overexpression of *TM6SF2* has the opposite effect.[3] In mice, hepatic knockdown of *TM6SF2* increases liver triglycerides (TG) and cholesteryl esters (CE), the main constituents of the hydrophobic core of very low-density lipoprotein (VLDL) particles.[2]

Keywords: Transmembrane 6 superfamily member 2; Triglycerides; Cholesterol esters; Arachidonic acid; Phosphatidylcholines; Non-alcoholic fatty liver disease; Lipogenesis; Genotype; Hepatocytes; Fatty acids.

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Hepatic steatosis, reduced hepatic TG secretion, and protection from CVD characterize animals with deletion of genes that are involved in phosphatidylcholine (PC) synthesis such as CTP: phosphocholine cytidylyltransferase alpha (*CTα*) and phosphatidylethanolamine N-methyltransferase (*PEMT*).[14,15] A similar phenotype also results from deletion of lysophosphatidylcholine acyltransferase 3 (*LPCAT3*), which decreases hepatic concentrations of polyunsaturated PCs.[16] PCs are the only phospholipids required for assembly of VLDL particles.[15] PC deficiency increases intrahepatic degradation of VLDL particles and thereby reduces their secretion.[15] Dietary restriction of choline, a precursor of PC, increases liver fat in humans.[17] Consumption of a methionine-choline-deficient diet, a widely used *in vivo* experimental model for NAFLD and NASH, reduces hepatic PC synthesis and plasma TGs and increases liver TGs without inducing insulin resistance.[15,18] In humans, a polymorphism in *PEMT*, which reduces PC synthesis, may be more frequent in participants with NAFLD[19–21] and in participants with non-alcoholic steatohepatitis (NASH)[22] than in those without, and characterizes participants who develop NAFLD on a choline-depleted diet.[23] Since these changes resemble those of carriers of the *TM6SF2* E167K gene variant,[2–13] we hypothesized that reduced PC synthesis might characterize carriers of the E167K variant.

In the present study, we profiled hepatic gene expression and measured concentrations of TGs, CEs, PCs and free fatty acids (FFA) in human liver biopsy samples from carriers (*TM6SF2*$^{EK/KK}$) and non-carriers (*TM6SF2*^{EE}) of the *TM6SF2* E167K variant. Since the *in vivo* results seemed to support our hypothesis, we directly measured incorporation of saturated (16:0, palmitic acid), monounsaturated (18:1, oleic acid), and polyunsaturated fatty acids (20:4, arachidonic acid) into TG, CE, and PC pools in *TM6SF2* knockdown HuH-7 cells. In addition, we measured circulating concentrations and composition of TGs, CEs and PCs in carriers and non-carriers of the gene variant.

Materials and methods

Study participants

Ninety participants were recruited from a cohort of patients undergoing laparoscopic bariatric surgery. Following a phone interview and a separate clinical study visit (*vide infra*), participants were eligible if they met the following inclusion criteria:

a. Age 18 to 75 years.
b. No known acute or chronic disease except for features of the metabolic syndrome based on medical history, physical examination and standard laboratory tests (complete blood count, serum creatinine, electrolyte concentrations).
c. Non-diabetic based on 2 h plasma glucose concentrations (⩽11 mmol/l) during a 75 g oral glucose tolerance test.
d. Alcohol consumption less than 20 g per day in women and less than 30 g per day in men.
e. No clinical or biochemical evidence of other liver disease, or clinical signs or symptoms of inborn errors of metabolism.
f. No use of drugs or toxins influencing liver steatosis.

Elevated liver enzymes (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) were not exclusion criteria. Participants with diabetes were excluded from the present study, as we wished to study the impact of the *TM6SF2* genotype at rs58542926 rather than that of insulin resistance/diabetes and antidiabetic drugs on the human liver lipidome. The effect of the latter was reported earlier in a larger (n = 125) group of participants, which included patients with diabetes.[24] The ethics committee of the Hospital District of Helsinki and Uusimaa approved the study, which was conducted in accordance with the Declaration of Helsinki. Each participant provided written informed consent after the nature and potential risks of the study had been fully explained.

Metabolic study

One week prior to surgery, all participants were invited to a clinical visit for metabolic characterization after an overnight fast. Anthropometric measurements (body weight, height and waist circumference) were taken, an intravenous cannula inserted in an antecubital vein and blood collected for measurement of HbA_{1c}, serum insulin and adiponectin, plasma glucose, LDL- and HDL-cholesterol, triglyceride, AST, ALT, ALP, GGT and albumin concentrations and for genotyping of *PNPLA3* rs738409 and *TM6SF2* rs58542926 as previously described.[24]

The participants underwent an oral glucose (75 g) tolerance test (OGTT).[25] HOMA-IR[26] and Matsuda insulin sensitivity index[27] were used as surrogates for insulin resistance. Body weight of the participants did not differ between the time of the metabolic study and surgery (128.9 ± 2.3 and 128.5 ± 2.3 kg; non-significant [NS]). The participants were divided into groups based on their *TM6SF2* rs58542926 genotype (E167K variant allele carriers, *TM6SF2*$^{EK/KK}$, n = 10 and non-carriers, *TM6SF2*^{EE}, n = 80).

Liver biopsies and liver histology

Routine wedge biopsies of the liver were taken immediately at the beginning of the surgery. Part of the biopsy was snap-frozen in liquid nitrogen for subsequent analysis of molecular lipids and gene expression; the other part was sent to an experienced liver pathologist (J.A.) for blinded analysis of liver histology according to the criteria proposed by Brunt *et al.*[28] Liver fat was quantified as the percentage of hepatocytes with macrovesicular steatosis.

Hepatic lipidomic and free fatty acid analysis

Hepatic lipidomic analyses of TGs, CEs and PCs were performed using a Q-TOF Premier (Waters, Milford, MA) quadrupole time-of-flight mass spectrometer combined with an Acquity Ultra Performance liquid chromatograph (LC) (Waters, Milford, MA). Liquid chromatography methods are described in the Supplementary material. Hepatic FFAs were analysed using an Agilent 6890 gas chromatograph equipped with a split/splitless injector (Agilent Technologies, Santa Clara, CA), cryogenic dual-stage modulator and time-of-flight mass spectrometer (Leco Corp., St. Joseph, MI, USA) as described in detail in Supplementary material. All analyses were blinded.

Hepatic RNA sequencing

Since the results (*vide infra*) suggested that TM6SF2 might be involved in synthesis of polyunsaturated lipids, we examined hepatic gene expression using RNA sequencing. For this, liver samples were available from 69 of the original cohort. An additional 25 participants were recruited using the same criteria as the original cohort. The characteristics of these participants (n = 94) are shown in Table S2. The samples were prepared using Illumina TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA) and sequencing was performed using a paired-end 101 base pair protocol on HiSeq2000 platform (Illumina, San Diego, CA). The methods for RNA sequencing, data processing and pathway analysis are described in detail in the Supplementary material.

Serum lipidomic analysis

Serum lipidomic analyses of TGs, CEs and PCs were analysed using an ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS). The UHPLC was a 1290 Infinity system (Agilent Technologies, Santa Clara, CA) and the mass spectrometer a 6550 iFunnel Q-TOF (Agilent Technologies, Santa Clara, CA). Details of the sample preparations and data preprocessing steps are given in Supplementary material.

In vitro experiments

Stably transduced and transiently silenced HuH-7 human hepatoma derived cells were used as *in vitro* hepatocyte models. The former was established by transducing HuH-7 cells with lentiviral particles expressing shRNA against *TM6SF2*

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(TRCN0000254085, Sigma-Aldrich, St. Louis, MO) and a control non-targeting shRNA (SHC002V, Sigma-Aldrich), and the latter by using siRNA against *TM6SF2* (SI04187946, Qiagen, Hilden, Germany) and a negative control (SI03650318, Qiagen) in HuH-7 cells. Silencing efficiency by quantitative polymerase chain reaction, lipid droplet quantification, and incorporation of tritiated fatty acids ([9,10-^{3}H(N)]-palmitic, [9,10-^{3}H(N)]-oleic and [5,6,8,9,11,12,14,15-^{3}H(N)]-arachidonic acid, PerkinElmer, Waltham, MA) into TGs, PCs and CEs by thin layer chromatography were analysed as described in detail in Supplementary material.

Statistical analyses

The Kolmogorov-Smirnov test was used to test the normality of continuous variables. The independent two-sample Student's *t* test and Mann-Whitney *U* test were used to compare normally and non-normally distributed data, respectively. Normally distributed data were reported in means ± standard error of means (SEM) while non-normally distributed data were reported as medians followed by interquartile ranges. Pearson's χ^2 test was used to evaluate if the distribution of categorical variables differed between the groups. Pearson's correlation coefficients were calculated as a measure of statistical dependence between two variables after log-transformation if necessary. Statistical analyses were performed by using R 3.1.1 (http://www.r-project.org/), IBM SPSS Statistics 22.0.0.0 version (IBM, Armonk, NY), and GraphPad Prism 6.0f for Mac OS X (GraphPad Software, La Jolla, CA). A two-sided *p* value of less than 0.05 indicated statistical significance.

The median and, after log2-transformation, the mean concentrations of all lipids were compared between groups based on *TM6SF2* genotype. Benjamini-Hochberg method was used for multiple hypotheses testing.[29] Differences in concentrations of TGs were illustrated by a heatmap, where the color intensity of each cell represents the log2-transformed ratio of mean values of an individual TG in the *TM6SF2*$^{EK/KK}$ group divided by the mean values in the *TM6SF2*^{EE} group. Fold-change of log2-transformed ratio of mean concentrations of lipids between the groups was visualized with respect to fatty acyl chain length and saturation.

For further details regarding the materials used, please refer to the CTAT table.

Results

Characteristics of the study groups

Clinical characteristics of the *TM6SF2* genotype groups are shown in Table 1. The groups were similar with respect to age, gender and BMI but liver fat was 2-fold higher in the *TM6SF2*$^{EK/KK}$ than the *TM6SF2*^{EE} group (Table 1). Histologic liver fat percentage correlated with the sum of all liver TGs measured by UHPLC-MS ($r = 0.81$, $p < 0.001$).

Fasting, 30 and 120 min glucose and insulin concentrations during the OGTT (Fig. 1) and the distribution of *PNPLA3* rs738409 genotype (Table 1) were comparable between the *TM6SF2* groups. The *TM6SF2*$^{EK/KK}$ tended to have higher prevalence of hepatic fibrosis compared to the *TM6SF2*^{EE} group (60% *vs.* 31%, $p = 0.09$).

Hepatic TGs

The increase in TGs in the *TM6SF2*$^{EK/KK}$ when compared to the *TM6SF2*^{EE} group was due to an increase in TG species with 48–54 carbons and 0–3 double bonds, *i.e.* TGs containing predominantly saturated and monounsaturated long-chain fatty acids (Fig. 2). Concentrations of TGs with 58–60 carbons and 6–11 double bonds, *i.e.* TGs containing predominantly polyunsaturated fatty acids were decreased in the *TM6SF2*$^{EK/KK}$ when compared to the *TM6SF2*^{EE} group (Fig. 2; Fig. S1; Table S1). Consequently, the fold-change of concentrations of TGs between the groups was significantly inversely correlated ($r = -0.27$, $p = 0.0033$) with

Table 1. Clinical characteristics of the study participants according to the ***TM6SF2*** genotype at rs58542926.

Total	***TM6SF2*^{EE} (n = 80)**	***TM6SF2*$^{EK/KK}$ (n = 10)**
Age (years)	45.2 ± 0.9	49.1 ± 2.8
Gender (% women)	71.3	70.0
BMI (kg/m^2)	45.4 ± 0.7	45.6 ± 1.7
Waist circumference (cm)	128.3 (120.0–140.0)	127.3 (117.4–135.4)
Liver fat (%)	13.8 ± 2.0	27.5 ± 9.5*
Necroinflammatory grade (0/1/2/3)	67/13/0/0	8/2/0/0
Fibrosis stage (0/1/2/3/4)	55/23/2/0/0	4/5/1/0/0
Fibrosis stage > 0 (%)	31	60 ($p = 0.09$)
fS-insulin (mU/L)	12.6 (6.9–18.8)	12.8 (7.7–17.7)
fP-glucose (mmol/L)	5.6 ± 0.1	5.7 ± 0.3
HbA_{1C} (%)	5.7 (5.5–6.0)	5.7 (5.5–6.1)
HbA_{1C} (mmol/mol)	39 (37–42)	39 (37–43)
HOMA-IR†	3.4 ± 0.2	3.6 ± 0.7
Matsuda index††	55.4 (36.0–104.9)	56.9 (42.2–74.3)
fS-adiponectin (μg/ml)	8.5 ± 0.5	10.1 ± 1.3
fP-total cholesterol (mmol/L)	4.3 ± 0.1	4.2 ± 0.2
fP-triglycerides (mmol/L)	1.24 (0.91–1.69)	1.34 (0.97–1.60)
fP-HDL cholesterol (mmol/L)	1.13 (0.96–1.37)	1.09 (0.96–1.55)
fP-LDL cholesterol (mmol/L)	2.6 ± 0.1	2.5 ± 0.2
P-AST (IU/L)	30 (25–37)	34 (30–41)
P-ALT (IU/L)	32 (24–45)	42 (28–58)
P-ALP (IU/L)	64 ± 2	70 ± 5
P-GGT (U/L)	28 (21–45)	50 (26–66)
P-albumin (g/L)	38.2 ± 0.3	37.9 ± 1.0
B-platelets (x 10^9/L)	252 ± 6	276 ± 35
PNPLA3 (n, CC/CG/GG)	41/35/4	6/3/1
TM6SF2 (n, CC/CT/TT)	80/0/0	0/9/1***

Data are in n, means ± SEM or median (25th–75th percentile), and statistical tests are Student's *t* test, Mann-Whitney *U* test and Pearson's χ^2-test, as appropriate. *$p \leqslant 0.05$. ***$p \leqslant 0.001$.

† Homeostasis model assessment of insulin resistance was calculated by formula: fS-Insulin x fP-Glucose/22.5 according to Matthews *et al.*[25]

†† Matsuda insulin sensitivity index was calculated from insulin and glucose concentrations measured at 0, 30 and 120 min during the oral glucose tolerance test as proposed by DeFronzo and Matsuda.[26]

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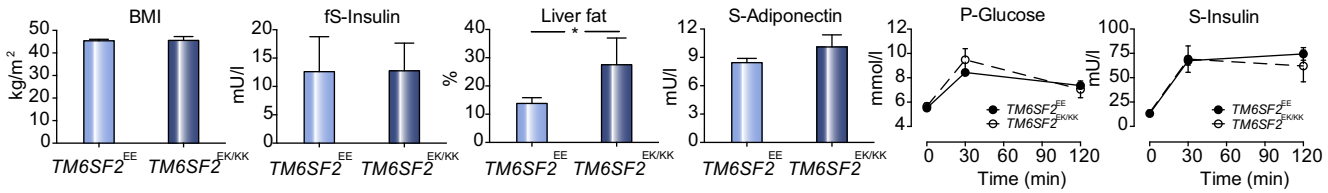

Fig. 1. Clinical characteristics of the groups based on the TM6SF2 genotype. BMI, fasting serum insulin, liver fat, serum adiponectin, plasma glucose concentrations during OGTT and serum insulin concentrations during OGTT. Light blue bars, black circles and solid lines, *TM6SF2*^{EE} (n = 80). Dark blue bars, open circles and broken lines, *TM6SF2*$^{EK/KK}$ (n = 10). Data are in means ± SEM or median (25th-75th percentile); Student's *t* test and Mann-Whitney *U* test, as appropriate; $^{*}p < 0.05$.

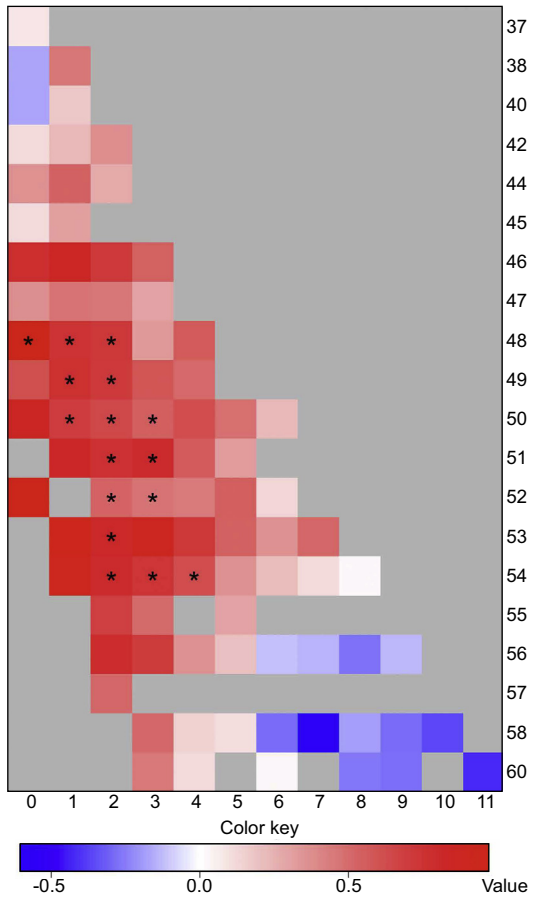

Fig. 2. Fold-change of hepatic triglycerides (TG) in the *TM6SF2*$^{EK/KK}$ group compared to the *TM6SF2*^{EE} group. The color key shows the log of the ratio between means of the groups of an individual TG. The brighter the red color, the greater increase of absolute concentration of the individual TG in the *TM6SF2*$^{EK/KK}$ compared to the *TM6SF2*^{EE} group. The y-axis denotes the number of carbons and the x-axis the number of double bonds. Student's *t* test; $^{*}p < 0.05$.

the number of double bonds in TGs (Fig. S1). *In vitro*, *TM6SF2* knockdown HuH-7 cells had 52% lower *TM6SF2* mRNA expression than control HuH-7 cells ($p < 0.001$) (Fig. 3A). Incorporation of palmitic acid (16:0, a saturated fatty acid) (+117%, $p < 0.01$) and oleic acid (18:1n-9, a monounsaturated fatty acid) (+39%, $p < 0.05$) into TGs in the *TM6SF2* knockdown HuH-7 cells were significantly increased, while that of arachidonic acid (20:4n-6, a polyunsaturated fatty acid) was significantly decreased (−34%, $p < 0.001$) (Fig. 3D). Transient silencing (−78%) of *TM6SF2* in HuH-7 cells induced similar changes in fatty acid incorporation into TGs (Fig. S2A).

Hepatic CEs

The concentration of total liver CEs was 20% higher in the *TM6SF2*$^{EK/KK}$ than the *TM6SF2*^{EE} group ($p < 0.05$) (Fig. 4). Among individual CE species, the concentration of CE(16:0) was significantly higher in the *TM6SF2*$^{EK/KK}$ group than the *TM6SF2*^{EE} group ($p < 0.05$), while those of CE(16:1), CE(18:1) and CE(18:2) tended to increase ($p = 0.07$, $p = 0.06$, and $p = 0.05$, Fig. 4). In *TM6SF2* knockdown HuH-7 cells, incorporation of palmitic acid (16:0) into CE was significantly (+149%, $p < 0.05$) and that of oleic acid (18:1) almost significantly (+76%, $p = 0.06$, Fig. 3E) increased. Transient silencing of TM6SF2 in HuH-7 cells induced similar changes in fatty acid incorporation into CEs (Fig. S2C).

Hepatic PCs

In the human liver samples, total PCs were significantly lower in the *TM6SF2*$^{EK/KK}$ than the *TM6SF2*^{EE} group (−11%, $p < 0.05$; Fig. 4). The fold-change of PCs between the groups was significantly inversely correlated with the number of double bonds ($r = -0.40$, $p < 0.001$) but not with the number of carbons in PCs. Thus, polyunsaturated PCs were responsible for the decrease in total PCs (Fig. 4). To determine whether the decrease in polyunsaturated PCs was secondary to an increase in liver TG, we plotted liver TGs against individual polyunsaturated PC concentrations. As shown in Fig. S3, for any given liver TG concentration, those of polyunsaturated PCs were significantly lower in the *TM6SF2*$^{EK/KK}$ than the *TM6SF2*^{EE} group.

In *TM6SF2* knockdown HuH-7 cells, the relative incorporation of arachidonic acid to PC was significantly lower than in control cells (−32%, $p < 0.05$, Fig. 3F), while incorporations of palmitic (+236%, $p < 0.001$) and oleic (+65%, $p < 0.05$) acids were increased (Fig. 3D). Transient silencing of *TM6SF2* expression in HuH-7 cells induced similar changes in fatty acid incorporation into PCs (Fig. S2B).

Hepatic FFA

The concentration of total hepatic FFA was significantly lower in the *TM6SF2*$^{EK/KK}$ (700 [595–822] nmol/g) than the *TM6SF2*^{EE} group (904 [755–1,098] nmol/g, $p < 0.01$). Among the individual FFA, concentrations of palmitic (16:0, 286 [254–346] *vs.* 383 [307–456] nmol/g, $p < 0.01$), stearic (18:0, 131 [125–188] *vs.* 195 [160–231] nmol/g, $p < 0.01$), oleic (18:1, 140 [131–168] *vs.* 191 [141–247] nmol/g, $p < 0.05$) and linoleic (18:2, 67 [50–81] *vs.* 84 [67–102] nmol/g, $p < 0.05$) acids were significantly lower in the *TM6SF2*$^{EK/KK}$ compared to the *TM6SF2*^{EE} group (Fig. 4). The relative proportion of arachidonic acid (20:4) in total FFA was significantly higher (+15%, $p < 0.05$) in the *TM6SF2*$^{EK/KK}$ compared to the *TM6SF2*^{EE} group (Fig. 4).

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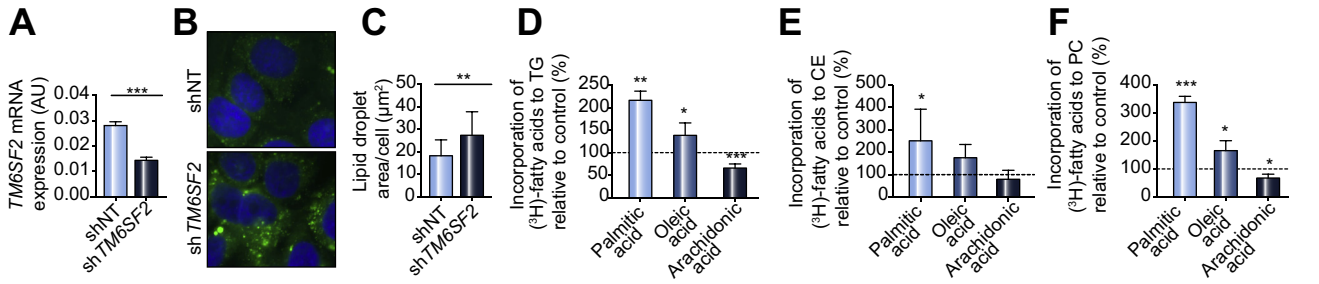

Fig. 3. *In vitro* experiments. A) *TM6SF2* mRNA expression in control shRNA (shNT) and *TM6SF2* shRNA (sh*TM6SF2*) expressing HuH-7 cells. The experiment was run twice in triplicates. B) Staining of lipid droplets by BODIPY® 493/503 in shNT and sh*TM6SF2* expressing HuH-7 cells and C) the quantification of lipid droplet area per cell. The data represent the average of 150 cells from each set. The relative incorporation of [^{3}H]-palmitic acid, [^{3}H]-oleic acid and [^{3}H]-arachidonic acid into triglycerides (D), cholesteryl esters (E) and phosphatidylcholines (F) expressed in percentage relative to control (n = 5). Data are shown as mean ± SD; Student's *t* test; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

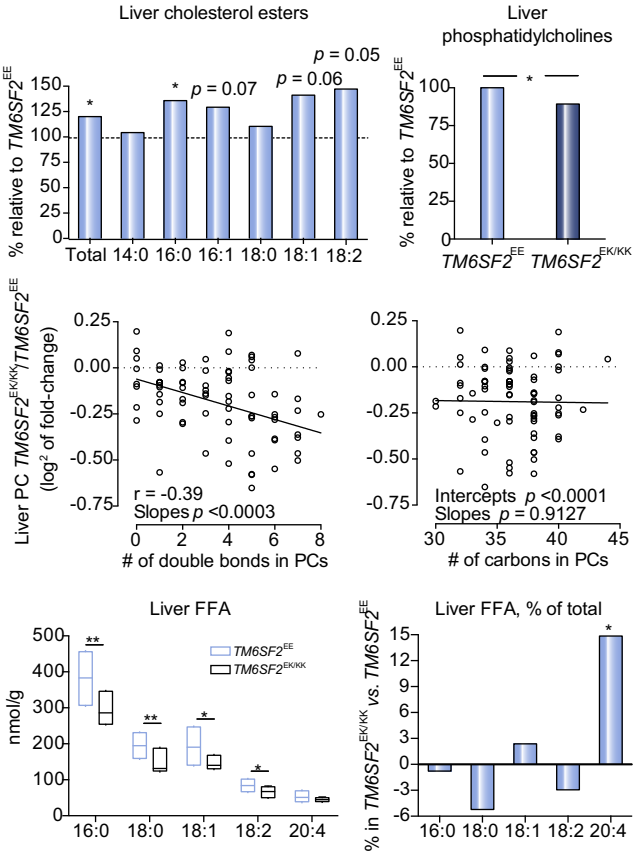

Fig. 4. Differences in hepatic cholesteryl esters (CE), phosphatidylcholines (PC) and free fatty acids (FFA) between the groups. Concentrations of total and individual liver CEs (top left panel) and total liver PCs (top right panel) in *TM6SF2*$^{EK/KK}$ relative to *TM6SF2*^{EE} group. The height of the bars represent% lipid concentration in *TM6SF2*$^{EK/KK}$ relative to *TM6SF2*^{EE} group. Linear regression between the number of double bonds (middle left panel) and carbons (middle right panel) in PCs and fold-change of liver PCs in the *TM6SF2*$^{EK/KK}$ *vs.* the *TM6SF2*^{EE} group (solid line). The intercepts and slopes denote deviation of this regression line from 0. Each circle denotes a different lipid. Concentrations of different liver FFA (bottom left panel) and liver FFA as a percentage of total (bottom right panel) in *TM6SF2*$^{EK/KK}$ and *TM6SF2*^{EE}. Data are in means ± SEM or median (25th–75th percentile); Student's *t* test and Mann-Whitney *U* test, as appropriate; $^*p < 0.05$, $^{**}p < 0.01$.

Serum TGs, PCs and CEs

In the serum, the fold-change of TGs in the *TM6SF2*$^{EK/KK}$ compared to the *TM6SF2*^{EE} group tended to inversely correlate with the number of double bonds ($p = 0.07$) and number of carbons ($p = 0.07$) in TGs (Fig. 5). The intercepts of the fold-change regression lines between the groups were highly significantly, lower than 0 in serum TGs and PCs according to number of double bonds ($p < 0.0001$ for both TGs and PCs) and carbons ($p < 0.0001$ for both), but not in serum CEs (Fig. 5).

Hepatic gene expression

TM6SF2 mRNA expression was significantly downregulated in variant allele carriers (number of rs58542926 minor (T) alleles *vs. TM6SF2* expression, $\beta = -0.41 \pm 0.11$, $p < 0.001$, Fig. S4). In pathway analyses, three pathways related to lipids, *i.e.* 'metabolism of lipids and lipoproteins', 'peroxisomal lipid metabolism' and 'triglyceride synthesis', were enriched with genes significantly correlated with *TM6SF2* (Fig. 6; Table S3). Among individual genes which were positively co-expressed with *TM6SF2* (and therefore decreased in *TM6SF2* E167K carriers) were those associated with fatty acid (fatty acid synthase [*FASN*], acetyl-CoA carboxylase beta [*ACACB*]) and triglyceride (diacylglycerol acyltransferase [*DGAT*]*1*, *DGAT2*) synthesis, and VLDL metabolism (apolipoprotein C-III [*APOC3*]), while those which were inversely co-expressed with *TM6SF2* included genes related to fatty acid oxidation (carnitine palmitoyltransferase 1 [*CPT1*]), peroxisome proliferator-activator receptors alpha and gamma (*PPARA*, *PPARG*) and eicosanoid synthesis (arachidonate 5-lipoxygenase [*ALOX5*]) (Fig. 6).

Discussion

To the best of our knowledge, this is, the first study to compare human hepatic lipid profiles between carriers and non-carriers of the *TM6SF2* E167K gene variant. Variant allele carriers had significantly higher hepatic TGs and CEs but lower total PCs due to a decrease in polyunsaturated PCs. Polyunsaturated fatty acids (PUFAs) were also deficient in serum and liver TGs but relatively enriched in liver FFA (Fig. 7). Consistent with this *in vivo* finding, using stably transduced and transiently silenced HuH-7 cells as

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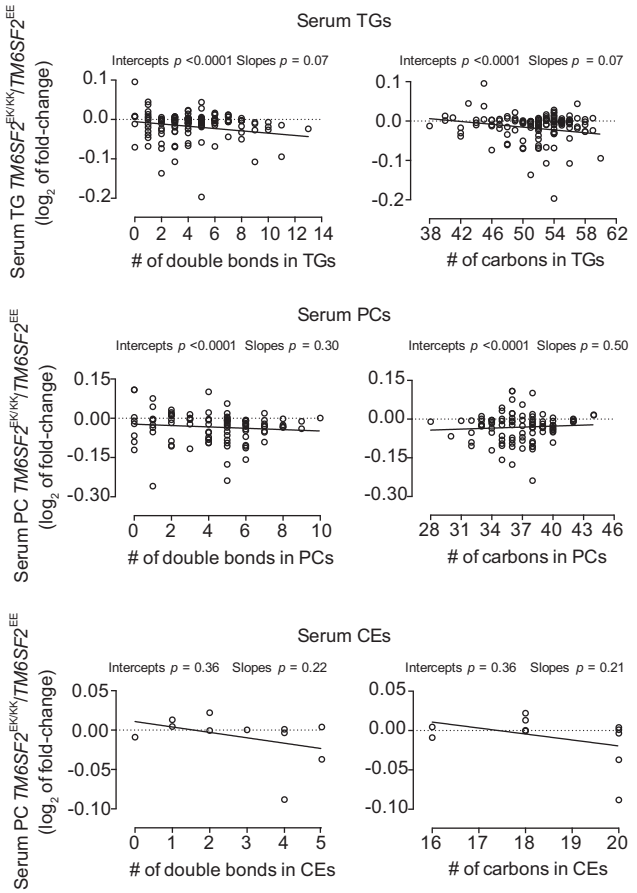

Fig. 5. Fold-change in serum triglycerides (TGs), phosphatidylcholines (PCs) and cholesteryl esters (CEs) in the *TM6SF2*$^{EK/KK}$ group compared to the *TM6SF2*^{EE} group. Linear regression between the number of double bonds (left panels) and carbons (right panels) in serum TGs (top), PCs (middle), and CEs (bottom) and fold-change of respective serum lipids in the *TM6SF2*$^{EK/KK}$ *vs.* the *TM6SF2*^{EE} group (solid line). The intercepts and slopes denote deviation of this regression line from 0. Each circle denotes a different lipid.

Metabolism of lipids and lipoproteins

APOC3, ***ECHS1***, ***SLC25A1***, ***FASN***, *APOE, ACOX3, DGAT1, DGAT2, APOA2, HADH, SLCO1B3, SLC25A17, ECI1, ACOX2, LPIN3, NEU4, MED11, ARSA, HSD11B1, GPD1, AKR1D1, BAAT, PHYH, ACADS, GPAM, SLC27A5, AGPAT3, SCARB1,* ***NPC1L1****, CYP17A1, CRAT, CYP8B1, MVK,* ***ACACB****, ABCC3, FDPS, CYP46A1, LSS, GPD2, GALC, FAR1, CYP7B1,* ***PPARA****, LBR, ARNTL, ASAH1, SGPP1, CPT1A, A2M,* ***PPARG****, OXCT1, GLIPR1, CLOCK,* ***ALOX5****, LRP2, SPTLC2, AGPS*

Triglyceride biosynthesis

SLC25A1, ***FASN***, *DGAT1, DGAT2, LPIN3, GPD1, GPAM, AGPAT3*

Peroxisomal lipid metabolism

ACOX3, SLC25A17, ACOX2, PHYH, CRAT, FAR1, AGPS

Fig. 6. Hepatic lipid-related gene expression pathways enriched with genes co-expressing with *TM6SF2*. Names of the pathways are in bold and names of the genes in italics. Blue color denotes genes correlating positively (*i.e.* decreasing in *TM6SF2* E167K carriers) and red color those correlating negatively with the expression of *TM6SF2*. Bold italics denote genes regulated by polyunsaturated fatty acids.[41,42,52–55]

in vitro hepatocyte models, *TM6SF2* knockdown led to reduced incorporation of PUFAs into PCs and TGs. In the human liver, *TM6SF2* was downregulated in variant allele carriers. Thus, downregulation of *TM6SF2* increases liver TG and CE and decreases PC content by impairing lipid synthesis from PUFAs.

To examine the effect of the *TM6SF2* E167K variant on human hepatic lipid content and composition, we divided the participants based on *TM6SF2* genotype (*TM6SF2*$^{EK/KK}$ *vs. TM6SF2*^{EE}). Carriers of this gene variant had 2-fold higher liver fat content but the groups did not differ with respect to HOMA-IR or insulin or glucose concentrations during the OGTT, or obesity. *PNPLA3* genotype, which influences hepatic lipid composition,[24,39] was also similar between the groups. Lack of features of insulin resistance between carriers and non-carriers of the *TM6SF2* allele is similar to the finding of Kozlitina *et al.* in three independent cohorts.[2] In keeping with several previous studies involving 300,[5] 361[6] and 691[7] participants, there was no difference in fasting plasma triglyceride concentrations between the *TM6SF2* genotype groups. However, in larger studies including 730[9], 1,201[12] and 4,587[2] participants, the variant allele has been associated with decreased circulating triglycerides. In the present study, variant allele carriers displayed deficiency of TGs and PCs in the circulation as compared to non-carriers in serum lipidomic analysis (Fig. 5). Deficiency of TGs and PCs in serum in the face of increased liver TG suggests defective hepatic lipid export, in line with previous data.[2,8–10,12]

In the liver of *TM6SF2* E167K variant carriers compared to non-carriers (Fig. 2, Fig. 4; Fig. S1) and *in vitro* in hepatocytes (Fig. 3D, F), PUFAs in both PCs and TGs were decreased while the proportion of polyunsaturated arachidonic acid in total liver FFA was increased (Fig. 4). These results imply that the *TM6SF2* E167K variant may regulate channeling of PUFAs into PC and TG synthesis. The first step in the incorporation of free PUFAs into these lipids is activation into a fatty acyl-CoA by an acyl-CoA synthetase (ACS) enzyme.[33] The number of ACS enzymes has rapidly increased and includes at least 26 isoforms.[34] These enzymes vary with respect to their substrate preference and subcellular localisation, and channel distinct fatty acids into different metabolic pathways.[33,34] For example, knockdown of acyl-CoA synthetase long-chain family member 3 (*ACSL3*), which is expressed in the liver and intestine and localised in the endoplasmic reticulum similarly to *TM6SF2*, decreases incorporation of unsaturated fatty acids into PC, causes PC deficiency, and inhibits secretion of VLDL in HuH-7 cells.[35]

Regarding the mechanisms by which PC deficiency and excess polyunsaturated FFA interfere with VLDL metabolism, assembly, secretion and degradation of VLDL needs to be considered. The assembly of VLDL has two major steps.[36] First, a nascent VLDL particle is formed in the ER, where it undergoes maturation to become a relatively lipid-poor small $VLDL_2$ particle.[36] This small particle is translocated to the Golgi complex and may be secreted as such, or undergoes a second lipidation step in which more TGs and CEs are added to the particle to form a highly-lipidated large $VLDL_1$ particle.[36]

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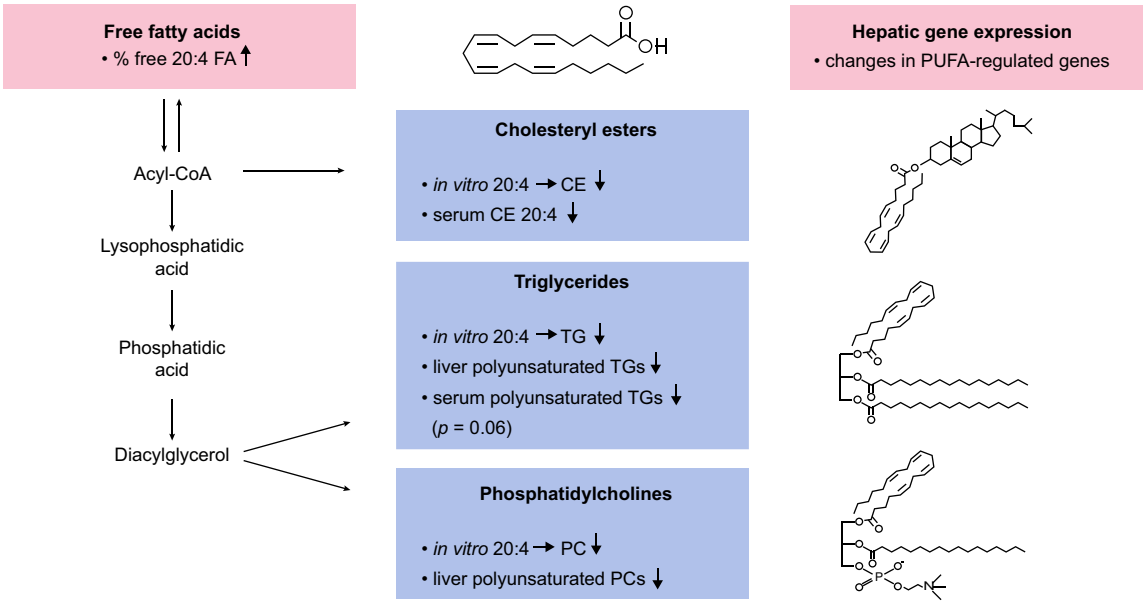

Fig. 7. Summary of changes in hepatic polyunsaturated fatty acid (PUFA) metabolism *in vivo* in *TM6SF2* E167K gene variant carriers, and *in vitro* in *TM6SF2* knockout HuH-7 cells. The percentage of hepatic free 20:4 fatty acid (FA) is increased *in vivo*. First step in synthesis of cholesteryl esters (CE), triglycerides (TG), and phosphatidylcholines (PC) is activation of free fatty acids into acyl-coenzyme A (CoA). These acyl-CoAs are esterified with cholesterol to form CEs. The *in vitro* incorporation of 20:4 into CEs is decreased, and concentrations of serum polyunsaturated CE are decreased *in vivo*. Acyl-CoAs are also utilized in *de novo* synthesis (*via* synthesis of lysophosphatidic acid, phosphatidic acid and diacylglycerol) of TGs and PCs. Incorporation of 20:4 into TGs and PCs is decreased *in vitro*, and hepatic concentrations of polyunsaturated TGs and PCs are decreased *in vivo*. Hepatic gene expression *in vivo* is characterized by changes in the expression of genes regulated by free PUFA. In summary, these *in vivo* and *in vitro* findings suggest that hepatic lipid synthesis from PUFA is impaired in *TM6SF2* E167K gene variant carriers.

In *TM6SF2* knockout mice, large VLDL particles are deficient in the circulation in the face of an unchanged or increased total number of particles secreted.[37] This was suggested to reflect a defect during the second lipidation step.[37] Consistent with these data in mice, in humans, large $VLDL_1$ particles are markedly deficient in the circulation in *TM6SF2* E167K variant allele carriers compared to non-carriers.[11] This may be caused by PC deficiency[15] or excess polyunsaturated FFA.[36,40] For example, in isolated mouse hepatocytes, reduced PC synthesis due to knockout of *PEMT* increases degradation and decreases secretion of particularly the highly-lipidated VLDL particles.[38] Polyunsaturated FFA increase VLDL degradation by a similar mechanism.[40] Thus, PC deficiency and the relative increase in polyunsaturated FFA in *TM6SF2* variant carriers might lead to accumulation of TGs and CEs *via* degradation of highly-lipidated VLDL, in keeping with the conclusion of Hobbs and coworkers.[37]

Hepatic expression of *TM6SF2* mRNA was decreased in E167K variant carriers (Fig. S4). Three lipid-related pathways were enriched with genes co-expressing with *TM6SF2* (Fig. 6). Among individual genes, expression of those related to lipogenesis and lipid export (*e.g. FASN*, *ACACB*, *DGAT2*, *DGAT1* and *APOC3*) was decreased, while expression of genes related to peroxisome proliferator-activated receptors, mitochondrial fatty acid oxidation, and eicosanoid synthesis (*e.g. PPARA*, *PPARG*, *CPT1*, *ALOX5*) was increased in the variant carriers (Fig. 6). Decreased expression of *DGAT1* and *DGAT2* in variant carriers is in line with previous *in vitro* data.[3] Interestingly, virtually identical changes in these genes are induced by PUFAs,[41] in part *via* transcriptional regulation by peroxisome proliferator-activated receptors[42] (Fig. 6). These findings are consistent with the *in vivo* and *in vitro* data suggesting impaired hepatic incorporation of PUFA to TGs, CEs and PCs, increased relative concentrations of arachidonic acid in hepatic FFA, and deficiency of TGs and PCs in serum in *TM6SF2* E167K gene variant carriers.

Although we observed PC deficiency both in the liver and in serum in humans carrying the *TM6SF2* E167K variant compared to non-carriers, no changes in hepatic PC concentrations were observed in *TM6SF2* knockout mice.[37] This could be due to species difference. The main finding of the present study, *i.e.* impaired hepatic lipid synthesis from PUFAs in *TM6SF2* E167K variant carriers, however, is entirely compatible with data in *TM6SF2* knockout mice displaying reduced percentage of 20:4 and 22:6 in total hepatic lipids.[37]

Even though the sample size in the present study was large considering the difficulty in obtaining human liver samples, the number of variant allele carriers was low given the variant allele frequency of 7%. The observed differences may thus underestimate true differences between carriers and non-carriers of the *TM6SF2* E167K gene variant. We had no detailed dietary records of the patients. This is a limitation as PC is also an important dietary component.[43] However, it is unlikely that genotype would affect intake of PC or its subsequent metabolism in gut lumen by bacteria to biologically active metabolites such as trimethylamine N-oxide.[44]

In keeping with human data of Liu *et al.*,[4] carriers of the *TM6SF2* E167K variant tended to have increased liver fibrosis compared to non-carriers (60 *vs.* 31%, $p = 0.09$). PC deficiency and the relative excess of arachidonic acid found in the present study may contribute to NASH and fibrosis by several mechanisms. PC deficiency induced by a methionine-choline-deficient diet induces lipid peroxidation, stellate cell activation and fibrosis in rats.[45] Excess arachidonic acid increases lipid peroxidation,

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and apoptosis,[46] and stellate cell collagen expression *in vitro*,[47] while arachidonic acid deficiency has opposite effects.[48] Arachidonic acid can be metabolized into proinflammatory eicosanoids,[33] which circulating concentrations are increased in participants with NASH.[49,50] Increased liver fat content *per se* may increase the risk of NASH, as evidenced by recent prospective liver biopsy data.[51]

In conclusion, hepatic synthesis of polyunsaturated fatty acid-containing lipids is impaired in *TM6SF2* E167K gene variant carriers and leads to deficiency of polyunsaturated PCs and excess polyunsaturated FFA in the human liver. These changes, based on abundant experimental data, could impair VLDL lipidation via increased degradation, and explain the phenotype characterizing *TM6SF2* E167K carriers *i.e.* hepatic steatosis, lack of circulating hypertriglyceridemia and an increased risk of NASH.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

PL – study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis. YZ, OPD, TT, PV – bioinformatics and statistical analyses; interpretation of data; critical revision of the manuscript for important intellectual content. PANH, VO – *in vitro* experiments; interpretation of data; critical revision of the manuscript for important intellectual content. TH, AA, AJ, ML, LA, JA, MOM, MO, LG – acquisition of data; critical revision of the manuscript for important intellectual content. ES, JMP, QMA – obtained funding; critical revision of the manuscript for important intellectual content. HY – study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; obtained funding; study supervision.

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Supplementary data

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Author names in bold designate shared co-first authorship

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