

Circulating triacylglycerol signatures and insulin sensitivity in NAFLD associated with the E167K variant in TM6SF2

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Background & Aims: The Glu167Lys (E167K) variant in the transmembrane 6 superfamily member 2 protein (*TM6SF2*) was recently shown to influence liver fat (LFAT) content. We aimed at studying how this variant influences circulating triacylglycerol (TAG) signatures and whether it influences hepatic or adipose tissue insulin sensitivity.

Methods: We genotyped 300 Finnish subjects for the E167K (rs58542926) variant in *TM6SF2* and for the I148M (rs738409) variant in the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) in whom LFAT was measured using ¹H-MRS and circulating lipids by UPLC-MS. We compared the plasma lipidome between E167K carriers (*TM6SF2*^{EK/KK}) and non-carriers (*TM6SF2*^{EE}), and between three groups of NAFLD: (i) carriers of the E167K but not of the I148M variant in *PNPLA3* ('*TM6SF2* NAFLD'), (ii) carriers of the I148M but not of the E167K variant ('*PNPLA3* NAFLD'), and (iii) non-carriers of either risk allele ('Non-risk NAFLD'). Hepatic and adipose tissue insulin sensitivity

were measured using the euglycemic hyperinsulinemic clamp technique combined with infusion of [³-³H]glucose in 111 subjects.

Results: The LFAT content was 34% higher in the *TM6SF2*^{EK/KK} (13.07 ± 1.57%) than in the *TM6SF2*^{EE} group (9.77 ± 0.58%, *p* = 0.013). The effect of insulin on glucose production and lipolysis were significantly higher in the *TM6SF2*^{EK/KK} than in the *TM6SF2*^{EE} group. Comparison of the three NAFLD groups with similar LFATs showed that both the '*TM6SF2* NAFLD' and '*PNPLA3* NAFLD' had significantly lower triglyceride levels and were characterized by lower levels of most common TAGs compared to the 'Non-risk NAFLD' group.

Conclusions: We conclude that the E167K variant in *TM6SF2* is associated with a distinct subtype of NAFLD, characterized by preserved insulin sensitivity with regard to lipolysis, hepatic glucose production and lack of hypertriglyceridemia despite a clearly increased LFAT content.

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Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANCOVA, analysis of covariance; ANOVA, analysis of variance; AST, aspartate aminotransferase; BP, blood pressure; BMI, body mass index; Cer, ceramide; FFA, free fatty acids; FP, fasting plasma; FS, fasting serum; GGT, gamma glutamyl transpeptidase; HbA1c, glycosylated haemoglobin 1c; HDL, high density lipoprotein; ¹H-MRS, proton magnetic resonance spectroscopy; HOMA-IR, homeostasis model assessment of insulin resistance; LFAT, liver fat; LC, lipid cluster; LDL, low-density lipoprotein; MetS, metabolic syndrome; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PNPLA3, patatin-like phospholipase domain-containing protein 3; TAG, triacylglycerol; *TM6SF2*, transmembrane 6 superfamily member 2; UPLC-MS, ultra-performance liquid chromatography mass spectrometry.

Introduction

Genetic factors contribute to the pathogenesis of non-alcoholic fatty liver disease (NAFLD). In 2008, an allele in the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), encoding I148M (rs738409 G-allele), was found to be highly significantly associated with NAFLD in three different ethnic groups [1]. This finding has subsequently been robustly replicated in over 50 studies, including eight genome wide association studies [2]. Recently, genetic variation in the transmembrane 6 superfamily member 2 protein (*TM6SF2*) at rs58542926 was shown to confer susceptibility to NAFLD, independent of genetic variation in *PNPLA3* at rs738409 in the Dallas Heart Study [3]. The *TM6SF2* variant, associated with NAFLD, is a guanine to adenine substitution, which replaces glutamate at residue 167 with lysine (E167K). The frequency of the allele variant was higher in individuals of European descent (7.2%, *n* = 882) than in any other ancestry group. *TM6SF2* variant allele carriers had significantly lower



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serum TAG concentrations than non-carriers in some but not all ethnic groups. The latter included European Americans [3]. Expression of the variant allele in cultured hepatocytes decreased production of the E167K TM6SF2 protein. Short hairpin RNA knockdown of *TM6SF2* in mice increased the triglyceride content three-fold and decreased VLDL secretion [3].

We have previously shown that rs738409 in *PNPLA3* is associated with a distinct circulating TAG signature, compared to obesity-associated NAFLD [4]. In the present study, we examined how the variant influences the circulating TAG signature as measured by UPLC-MS and whether it influences directly measured hepatic or adipose tissue insulin sensitivity. We also compared the plasma lipidome between three groups with NAFLD: those carrying only the E167K variant in *TM6SF2* but not the I148M allele in *PNPLA3* ('*TM6SF2* NAFLD'), another carrying only the I148M risk allele in *PNPLA3* ('*PNPLA3* NAFLD') and those carrying neither of these risk alleles ('Non-risk NAFLD').

Materials and methods

Subjects and study design

Metabolic studies were conducted at the University Central Hospital of Helsinki, Finland. The subjects ($n = 300$) were recruited using the following criteria: (a) age 18 to 75 years; (b) no known acute or chronic disease except for obesity or type 2 diabetes based on medical history, physical examination and standard laboratory tests (blood counts, serum creatinine, thyroid-stimulating hormone, electrolyte concentrations) and electrocardiogram; (c) alcohol consumption less than 20 g per day. Elevated liver enzymes (serum alanine aminotransferase [ALT], and aspartate aminotransferase [AST]) were not exclusion criteria. However, subjects with clinical or biochemical evidence of hepatitis B or C, autoimmune hepatitis, or with clinical signs or symptoms of inborn errors of metabolism or a history of use of toxins or drugs associated with liver steatosis were excluded. Eighty-two subjects had type 2 diabetes. Patients were excluded if they used thiazolidinediones or were pregnant. Lipidomics data on a larger study group have been previously reported [4]. The present study included subjects from whom DNA could be obtained for genotyping of *PNPLA3* at rs738409 and *TM6SF2* at rs58542926. The study protocol was approved by the ethics committee of the Helsinki University Central Hospital. Each participant signed an informed consent form for participation in the metabolic study and another form, permitting blood sampling and analysis of polymorphisms in genes related to the LFAT content.

In eligible subjects, a blood sample was taken after an overnight fast for lipidomic analyses (see below) and for measurements of glucose, insulin, total TAGs, total and HDL cholesterol, AST, ALT, and gamma glutamyl transpeptidase (GGT) concentrations. A blood sample was also obtained at this visit or by inviting subjects to a separate visit, for the isolation of DNA and genotyping. In each subject, the LFAT content was measured (see below). Direct measurements of hepatic and adipose tissue insulin sensitivity using 6-h infusions of [$^3\text{-}^3\text{H}$]glucose and glucose and insulin were performed in 111 of the subjects (see below).

Lipidomic analysis with UPLC-MS

An unfrozen plasma sample was extracted for lipidomic analysis (see below). An established platform, based on Acquity Ultra Performance LC™ coupled to a quadrupole time-of-flight mass spectrometer (UPLC-MS) was used to analyse the plasma samples [5].

An aliquot (10 μL) of the plasma sample was diluted with 10 μL of 0.15 M (0.9%) sodium chloride, and 10 μL of internal standard mixture 1A was added. This mixture contained PC(17:0/0:0), PC(17:0/17:0), PE(17:0/17:0), PG(17:0/17:0), Cer(d18:1/17:0), PS(17:0/17:0), and PA(17:0/17:0) (Avanti Polar Lipids, Inc., Alabaster, AL) as well as mono-triglycerides (17:0/0:0/0:0), di-triglycerides(17:0/17:0/0:0) and TAG(17:0/17:0/17:0). The lipids were extracted using a mixture of HPLC-grade chloroform and methanol (2:1; 100 μL). The lower phase (60 μL) was collected and 10 μL of an internal standard mixture containing labelled PC (16:1/0:0- D_3), PC(16:1/16:1- D_6), and TAG(16:0/16:0/16:0- $^{13}\text{C}_3$) was added.

The extracts were analysed on a Waters Q-ToF Premier mass spectrometer combined with an Acquity Ultra Performance LC™. The column was an Acquity UPLC™ BEH C18 2.1 \times 100 mm with 1.7 μm particles, kept at 50 °C. The solvent system included (A) ultrapure water (1% 1M NH_4Ac , 0.1% HCOOH) and (B) LC/

MS grade acetonitrile/isopropanol (1:1, 1% 1M NH_4Ac , 0.1% HCOOH). The gradient started from 65% A / 35% B, reached 80% B in 2 min, 100% B in 7 min and remained there for 7 min. The flow rate was 0.400 ml/min and the injected amount was 2.0 μL (Acquity Sample Organizer, at 10 °C). Reserpine was used as the lock spray reference compound. Lipid profiling was carried out using electrospray ionization in positive ion mode and data were collected at a mass range of m/z 300–1200 with scan duration of 0.2 s.

The data processing, including alignment of peaks, peak integration, normalization and identification was done by using the MZmine 2 software [6] and lipid identification was based on an internal spectral library or on *de novo* identification using tandem MS [5]. Data were normalized by using one or more internal standards, representative of each class of lipid class present in the samples: the intensity of each identified lipid was normalized by dividing it with the intensity of its corresponding standard and multiplying it by the concentration of the standard. All monoacyl lipids except cholesterol esters, such as monoacylglycerols and monoacylglycerophospholipids, were normalized with PC(17:0/0:0), all diacyl lipids except ethanolamine phospholipids were normalized with PC(17:0/17:0), all ceramides with Cer(d18:1/17:0), all diacyl ethanolamine phospholipids with PE(17:0/17:0), and TAG and cholesterol esters with TAG(17:0/17:0/17:0). Other (unidentified) molecular species were normalized with PC(17:0/0:0) for retention times <300 s, with PC(17:0/17:0) for a retention time between 300 s and 410 s, and with TAG(17:0/17:0/17:0) for longer retention times. For further identification of unknown lipids, please see [Supplementary Materials and methods](#) for further details.

LFAT content

The LFAT content was measured by proton magnetic resonance spectroscopy (^1H -MRS) as previously described and validated against histologic measurement of LFAT [7]. In a few subjects ($n = 36$), LFAT was measured using liver biopsy. The fat content of the liver biopsy specimens (the percentage of hepatocytes with macrovesicular steatosis) was determined using haematoxylin & eosin staining and converted to ^1H -MRS LFAT percentage units, as previously described [7]. NAFLD was defined as LFAT ≥ 55.6 mg triglyceride per g of liver tissue or $\geq 5.56\%$ of liver tissue weight [8].

Genotyping

Genomic DNA was extracted from whole blood. Genotyping was performed using Taqman PCR method (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. ABI Prism Sequence Detection Systems ABI 7900HT (Applied Systems) was used for post PCR allelic discrimination by measuring allele-specific fluorescence. The success rate for genotyping was >95%. Genotyping was performed twice in two independent analyses and the concordance rate was 100%. The genotypes were in Hardy-Weinberg equilibrium.

Hepatic and adipose tissue insulin sensitivity

Insulin sensitivities of hepatic glucose production and peripheral glucose utilization as well as the antilipolytic effect of insulin were assessed by the euglycemic hyperinsulinemic clamp technique combined with the infusion of [$^3\text{-}^3\text{H}$] glucose as previously described [9]. Because hepatic glucose production is more sensitive to suppression by insulin than stimulation of muscle glucose uptake, we used a low insulin infusion rate (0.3 mU/kg-min) to accurately quantify interindividual variation in hepatic insulin sensitivity. These measurements were available from 111 subjects. The rate of glucose endogenous production and the rate of glucose disposal were calculated using Steele's non-steady-state equations. Since insulin clearance is altered by a fatty liver [7], hepatic insulin sensitivity was calculated by dividing the percentage suppression of hepatic glucose production by the mean serum insulin concentration (mU/L). Adipose tissue insulin sensitivity was calculated by dividing the percentage suppression of serum-free fatty acids (S-FFA) by insulin by the mean serum insulin concentration (mU/L) [9].

Other analytical procedures and measurements

Body weight was recorded to the nearest 0.1 kg using a calibrated weighting scale with subjects standing barefoot and wearing light indoor clothing. Waist circumference was measured midway between the spina iliaca superior and the lower rib margin. Body height was recorded to the nearest 0.5 centimetre. Blood pressure, fasting plasma (FP) glucose, fasting serum (FS) insulin, FS-LDL cholesterol, total serum cholesterol, FS-HDL cholesterol, FS-TAG, AST, ALT, alkaline phosphatase (ALP), and GGT concentrations were measured as previously described

[10]. Homeostasis model assessment of insulin resistance (HOMA-IR) was estimated by using the formula: $HOMA-IR = fS-insulin (mU/L) \times fP-glucose (mmol/L) / 22.5$ [11]. The 'non-alcoholic steatohepatitis (NASH) score' was calculated as previously reported [12].

Statistical analyses

All data were tested for normality using the Kolmogorov-Smirnov test. Normally distributed data are shown as mean \pm SEM and non-normally distributed data are shown as median followed by the 25th and 75th quartiles. The unpaired *t* test and the Mann-Whitney test were used to compare normally and non-normally distributed data, respectively. One-way analysis of variance (ANOVA) was used to compare three NAFLD groups. The least square difference test was used for post hoc analyses. Non-normally distributed data were used after log10 transformation. Analysis of covariance (ANCOVA) was used to compare slopes and intercepts of regression lines of the associations between insulin sensitivities and LFAT content in different genotype carriers. The statistical analyses were performed by using R (<http://www.r-project.org/>) and STATA version 13.1 for Mac (StataCorp LP, College Station, TX). Figures were produced by R and GraphPad Prism 6 for Mac (GraphPad Software Inc., San Diego, CA). A two-sided *p* value of less than 0.05 was considered statistically significant.

Assessment of abundances of TAG species

Mean values and standard errors of abundances of plasma TAG molecular species were calculated. After log2 transformation, the mean values of TAG abundances were compared between the *TM6SF2* variant and wild type by using Student's *t* tests. Multiple comparisons were corrected by using the Benjamini-Hochberg's method [13]. Comparisons of TAG abundances were illustrated by heatmaps, which show fatty acid chain lengths and number of double bonds. The log2 transformed ratios of TAG values of the variant, divided by non-variant allele carriers, were visualized by each cell in the heatmap. R Package, metadar (<http://code.google.com/p/metadar>) was used for data analysis.

Analysis of lipidomics data

Groups of lipids with similar profiles were identified by using Bayesian model-based clustering as previously described [4].

Results

Comparison of *TM6SF2* gene variant carriers (*TM6SF2*^{EK/KK}) to non-carriers (*TM6SF2*^{EE})

The allele frequency of E167K was 7.2% with 0.7% being homozygous and 13% heterozygous for the K-allele. For comparison, the allele frequency of the I148M variant in *PNPLA3* was 27.8% with 6.0% being homozygous and 43.7% heterozygous. Age, gender, body weight, BMI, fasting plasma glucose concentrations and *PNPLA3* genotypes were similar between the *TM6SF2*^{EK/KK} and *TM6SF2*^{EE} groups (Table 1). Carriers of the *TM6SF2* gene variant (*TM6SF2*^{EK/KK}) had 34% higher mean LFAT (mean \pm SE: 13.07 \pm 1.57% vs. 9.77 \pm 0.58%, *p* value = 0.013, Fig. 1) or 65% higher median LFAT (median [25th–75th percentiles]: 11.2% [4.4–19.7] vs. 6.8% [2.1–15.6], *p*-value = 0.03) than those lacking the variant (*TM6SF2*^{EE}). Serum TAG, LDL, and HDL cholesterol and liver enzyme concentrations were comparable between the groups (Table 1).

We also compared patients with type 2 diabetes who were *TM6SF2* variant carriers (*TM6SF2*^{EK/KK}) to non-carriers (*TM6SF2*^{EE}). The *TM6SF2*^{EK/KK} group (*n* = 10) compared to the *TM6SF2*^{EE} group (*n* = 72) had significantly higher LFAT content (mean \pm SE: 19.4% \pm 3.0 vs. 13.4% \pm 1.1, *p* = 0.02) and were almost significantly less obese (32.0 kg/m² \pm 1.7 vs. 36.3 kg/m² \pm 1.0, *p* = 0.059). The groups were comparable with respect to age (54 yrs \pm 3 vs. 52 yrs \pm 1), gender (80% vs. 60% men, *p* = 0.37), fasting

Table 1. Characteristics of study subjects according to the *TM6SF2* E167K genotype.

Characteristic	<i>TM6SF2</i> ^{EK/KK} (n = 41)	<i>TM6SF2</i> ^{EE} (n = 259)	<i>p</i> value
Age (years)	50 \pm 2	47 \pm 1	n.s.
Gender (% women)	39	37	n.s.
Type 2 diabetes (%)	24	28	n.s.
BMI (kg/m ²)	32.5 \pm 1.0	33.7 \pm 0.5	n.s.
fP-glucose (mmol/L)	6.0 (5.4-7.5)	5.9 (5.4-7.0)	n.s.
fS-insulin (mU/L)	10.0 (7.0-16.6)	11.0 (6.5-15.8)	n.s.
HOMA-IR	2.9 (1.8-4.9)	3.0 (1.7-4.9)	n.s.
HbA _{1c} (%)	5.8 (5.5-6.6)	5.8 (5.5-6.3)	n.s.
Systolic BP (mmHg)	133 \pm 2	133 \pm 1	n.s.
Diastolic BP (mmHg)	84 \pm 1	83 \pm 1	n.s.
fS-triglycerides (mmol/L)	1.40 (1.00-1.75)	1.48 (1.06-2.11)	n.s.
fS-HDL cholesterol (mmol/L)	1.29 (1.11-1.61)	1.21 (1.04-1.53)	n.s.
fS-LDL cholesterol (mmol/L)	2.91 (2.37-3.50)	2.88 (2.25-3.50)	n.s.
FFA (μ mol/L)	628 (464-812)	635 (495-809)	n.s.
S-AST (IU/L)	30 (24-37)	29 (23-44)	n.s.
S-ALT (IU/L)	34 (23-47)	34 (23-55)	n.s.
S-ALP (IU/L)	73 (64-136)	78 (64-105)	n.s.
S-GGT (U/L)	35 (19-55)	35 (20-63)	n.s.
<i>PNPLA3</i> (<i>PNPLA3</i> ^{II} / <i>PNPLA3</i> ^{IM/MM}) (n)	19/22	130/129	n.s.

Data are in n (%), mean \pm SEM or median (25th–75th percentile), as appropriate.

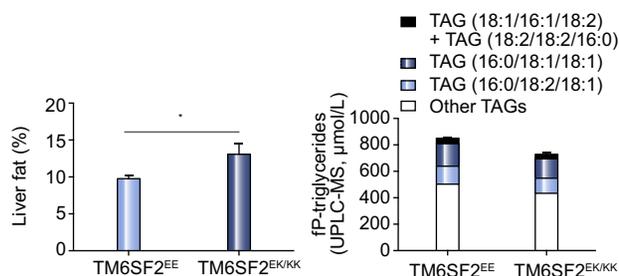


Fig. 1. Liver fat (%) and distribution of triglyceride species in plasma. Liver fat (%) was measured by ¹H-MRS (panel on the left) and the distribution of triglyceride species in plasma by UPLC-MS (panel on the right) in carriers of the E167K variant in *TM6SF2* (*TM6SF2*^{EK/KK}) and in non-carriers (*TM6SF2*^{EE}). Data are shown as mean \pm SEM (**p* < 0.05).

serum TAGs (2.1 mmol/L \pm 0.3 vs. 2.2 mmol/L \pm 0.2), insulin (14.1 mU/L \pm 1.7 vs. 16.4 mU/L \pm 1.3), HOMA-IR (6.7 \pm 1.0 vs. 6.0 \pm 0.5) and the *PNPLA3* genotype.

Serum lipidome in *TM6SF2*^{EK/KK} vs. *TM6SF2*^{EE}

Using the UPLC-MS based analytical platform, a total of 411 molecular lipids were measured and 157 identified. Total fasting serum (fS)-TAGs, measured enzymatically in the clinical laboratory (1.81 \pm 0.077 mmol/L, *n* = 300), were highly correlated with the sum of plasma TAGs identified by UPLC-MS (0.83 \pm 0.026 mmol/L, *r* = 0.88, *p* < 0.001). The lipidomic platform data were decomposed into 9 lipid clusters (LCs), which to a large

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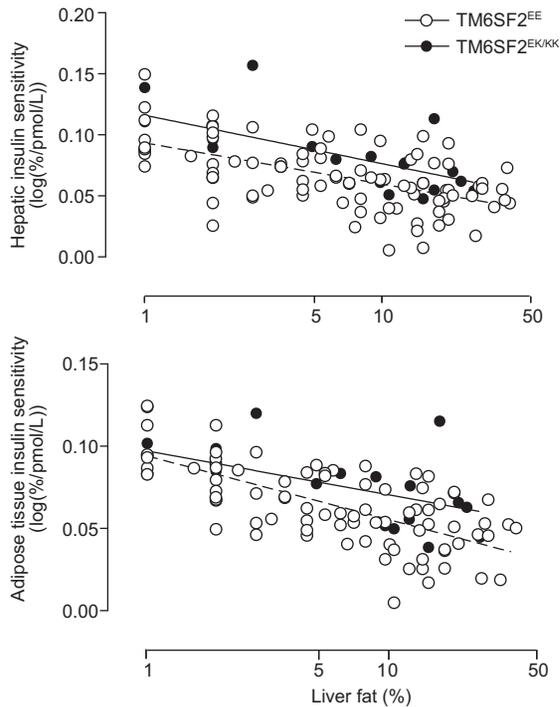


Fig. 2. Liver fat (%) and insulin sensitivity in TM6SF2EK/KK and TM6SF2EE groups. Relationships between liver fat and hepatic (top panel) and adipose tissue insulin sensitivity (bottom panel) were compared between the TM6SF2^{EK/KK} (closed circles) and TM6SF2^{EE} (open circles) groups. There were no differences between the slopes of these regression lines between the two groups. The intercept of the regression line relating liver fat and insulin sensitivity of hepatic glucose production was significantly ($p = 0.002$) lower in the TM6SF2^{EK/KK} than the TM6SF2^{EE} group. Similarly, the intercept of the regression line relating liver fat and insulin sensitivity of lipolysis was significantly ($p = 0.012$) lower in the TM6SF2^{EK/KK} than the TM6SF2^{EE} group. These data show that for any given liver fat content, the abilities of insulin to suppress hepatic glucose production and inhibit lipolysis were significantly better in the TM6SF2^{EK/KK} than the TM6SF2^{EE} group.

extent adhered to different lipid functional or structural groups (Supplementary Table 1). TAGs were particularly enriched in LC1 and LC6. LC1 was slightly lower ($p = 0.07$) in TM6SF2^{EK/KK} as compared to the TM6SF2^{EE} group. Plasma TAG composition did not differ between the groups (Fig. 1).

Insulin sensitivity

ANCOVA showed that there was no significant interaction between the TM6SF2 genotype and insulin sensitivity with regard to hepatic glucose production ($p = 0.87$) or lipolysis ($p = 0.31$). However, for any given LFAT content, as judged from significant differences in the intercepts between the regression lines, hepatic insulin sensitivity of glucose production ($p = 0.002$) and adipose tissue lipolysis ($p = 0.012$) were significantly higher in the TM6SF2^{EK/KK} than the TM6SF2^{EE} group (Fig. 2).

Comparison of NAFLD groups

Table 2 shows those subjects, who had NAFLD ($n = 154$) of all 300 subjects. The 'TM6SF2 NAFLD', 'PNPLA3 NAFLD', and 'Non-risk NAFLD' groups were comparable with respect to age, gender and BMI (Table 2). LFAT content was similar in all three groups

Table 2. Characteristics of the three NAFLD groups. This comparison includes all subjects with NAFLD (liver fat $\geq 5.56\%$) ($n = 154$).

Total	TM6SF2 NAFLD (n = 13)	PNPLA3 NAFLD (n = 77)	Non-risk NAFLD (n = 64)
Age (years)	49.5 \pm 13.3	47.5 \pm 11.2	47.6 \pm 10.7
Gender (% women)	46	51	53
Type 2 diabetes (%)	38	45	33
BMI (kg/m ²)	32.6 \pm 6.1	35.7 \pm 7.8	33.5 \pm 5.8
fP-glucose (mmol/L)	6.2 (5.6-10.7)	6.2 (5.7-7.7)	6.6 (5.7-8.0)
fS-insulin (mU/L)	14.2 (10.4-19.0)	14.0 (9.0-19.2)	13.6 (10.6-19.7)
HOMA-IR	3.8 (2.8-5.4)	3.7 (2.5-5.4)	4.5 (2.8-6.1)
HbA _{1c} (%)	6.0 (5.6-7.4)	6.0 (5.6-6.9)	6.0 (5.7-7.7)
Systolic BP (mmHg)	135 \pm 18	135 \pm 16	134 \pm 15
Diastolic BP (mmHg)	86 \pm 11	85 \pm 9	83 \pm 9
fS-triglycerides (mmol/L)	1.41 (1.06-1.97)*	1.60 (1.22-2.17)*†	1.92 (1.42-2.75)
fS-HDL cholesterol (mmol/L)	1.29 (1.13-1.42)	1.12 (1.00-1.34)	1.14 (0.94-1.27)
fS-LDL cholesterol (mmol/L)	2.87 (2.07-3.48)	2.82 (2.23-3.46)	3.20 (2.57-3.73)
fS-FFA (μ mol/L)	625 (486-722)	628 (541-808)	718 (583-823)
S-AST (IU/L)	33 (27-43)	39 (30-53)*†	31 (24-43)
S-ALT (IU/L)	42 (33-52)	51 (34-88)*†	38 (28-62)
S-ALP (IU/L)	79 (55-126)	71 (65-93)	83 (64-132)
S-GGT (U/L)	46 (25-78)	48 (32-66)	34 (24-77)
LFAT (%)	15.8 (11.2-23.3)	15.6 (9.8-21.0)	11.9 (8.2-20.5)
NASH score [#]	-1.80 (-1.92--1.41)	-0.88 (-1.31-0.03)	-1.70 (-2.00--1.47)

Data are in n (%), mean \pm SEM or median (25th–75th percentile). † $p < 0.05$ for one-way ANOVA; * $p < 0.05$ for LSD *post hoc* test compared with the 'Non-NAFLD risk'. #NASH score estimate based on AST, PNPLA3 genotype and fS-insulin [12].

(Table 2; Fig. 3, bottom panel on the left). Serum total TAGs were significantly lower in the 'TM6SF2 NAFLD' and 'PNPLA3 NAFLD' groups than in the 'Non-risk NAFLD' group (Table 2; Fig. 3, bottom panel on the right). Other lipid concentrations were comparable between the groups.

We compared TAG concentrations measured by UPLC-MS between the 'TM6SF2 NAFLD' and the 'Non-risk NAFLD' groups. The differences in circulating TAGs between the 'TM6SF2 NAFLD' and 'Non-risk NAFLD' resembled those observed between the 'PNPLA3 NAFLD' and 'Non-risk NAFLD' groups (Fig. 3, upper panel on the right; Fig. 4). The differences in circulating TAGs between the 'TM6SF2^{EK/KK} NAFLD' and the 'Non-risk NAFLD' groups were largely confined to the most abundant TAG species i.e. TAG(16:0/18:2/18:1) and TAG(16:0/18:1/18:1) (Fig. 4 and Supplementary Fig. 1). These molecular species were also significantly deficient in the 'PNPLA3 NAFLD' as compared to the 'Non-risk NAFLD' group (Figs. 3 and 4).

Discussion

The present study replicates the association between the TM6SF2 variant, encoding E167K, and increased LFAT content. We extended previous data by examining how the variant (and

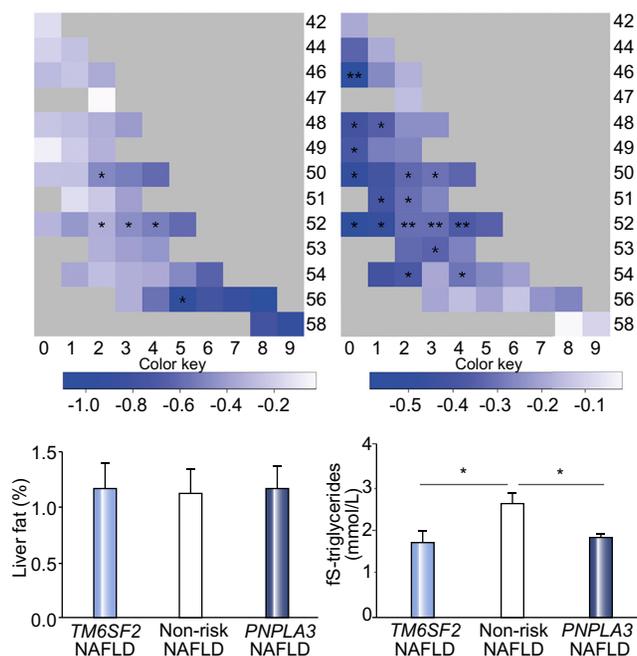


Fig. 3. Plasma TAGs, liver fat (%) and serum total TAGs. Concentrations of plasma TAGs were compared between the 'TM6SF2 NAFLD' and 'Non-risk NAFLD' (upper panel on the left) and between 'PNPLA3 NAFLD' and 'Non-risk NAFLD' (upper panel on right) groups. Liver fat was comparable between the three groups (bottom panel on the left), while serum total TAGs were significantly lower in the 'TM6SF2 NAFLD' and the 'PNPLA3 NAFLD' as compared to the 'Non-risk NAFLD' group (bottom panel on the right). The color code denotes the log of the ratio between means of the groups for an individual TAG. The y-axes denote the number of carbons, and the x-axes the number of double bonds. The darker the blue color, the greater the decrease in the 'TM6SF2 NAFLD' as compared to the 'Non-risk NAFLD' group (upper panel on the left) or between the 'PNPLA3 NAFLD' as compared to the 'Non-risk NAFLD' group (upper panel on the right). The significant differences in individual TAGs are indicated (**p* < 0.05, ***p* < 0.01). (This figure appears in color on the web.)

increased liver fat) influences the circulating TAG profile and directly measured insulin sensitivity. We also analysed how the *TM6SF2* variant allele *per se*, when associated with NAFLD, influences circulating TAGs by comparing lipidomics profiles between three groups with NAFLD and similar amount of LFAT. These were NAFLDs, associated with the *TM6SF2* E167K but not with the *PNPLA3* I148M variant allele ('*TM6SF2* NAFLD'), NAFLD associated with the *PNPLA3* but not with the *TM6SF2* E167K allele ('*PNPLA3* NAFLD') and NAFLD associated with neither ('Non-risk NAFLD'). We found the *TM6SF2* variant to be metabolically 'silent' as individuals carrying this variant were characterized by preserved insulin sensitivity with regard to lipolysis and hepatic glucose production despite having a clearly increased liver fat content. Comparison of NAFLD subgroups, defined by genetic background of NAFLD, revealed lower levels of the major circulating TAGs in both '*TM6SF2* NAFLD' and '*PNPLA3* NAFLD' compared to the 'Non-risk NAFLD' group.

In the present study, the allele frequency of the *TM6SF2* E167K variant was 7.2%. This frequency is identical to that reported in the Dallas Heart study (7.2% in European Americans) [3]. Consistent with the latter study in European Americans, mean liver fat content, measured by the same ¹H-MRS technique, was 34% increased in carriers of the E167K minor allele in the present study.

As in the Dallas Heart Study in European American subjects (n = 882), we found no significant differences in serum total TAG, HDL, and LDL cholesterol concentrations between *TM6SF2* variant allele carriers and non-carriers. Similarly, in a very recent study in 5643 Norwegians, the *TM6SF2* variant was associated with slightly lower total cholesterol but with no change in total TAG or HDL cholesterol concentrations [14]. However, in larger cohorts, in which lipid concentrations but not LFAT content have been measured, serum TAGs and LDL cholesterol concentrations have been significantly lower in carriers of the E167K allele as compared to non-carriers [3]. In mice, a selective knockdown of *TM6SF2* in the liver by short hairpin RNAs decreased protein lev-

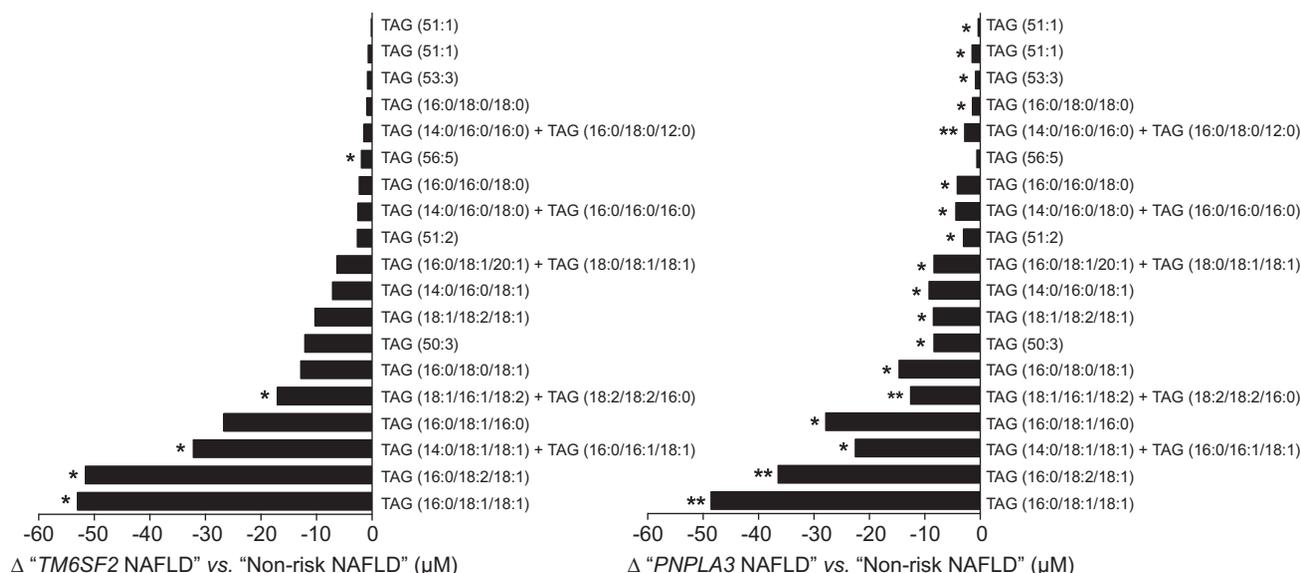


Fig. 4. Distribution of differences in plasma TAG species between 'TM6SF2 NAFLD', 'PNPLA3 NAFLD', and 'Non-risk NAFLD' groups. The x-axis indicates mean differences of absolute TAG concentrations between the '*TM6SF2* NAFLD' (panel on the left), '*PNPLA3* NAFLD' (panel on the right) and 'Non-risk NAFLD' groups (**p* < 0.05, ***p* < 0.01).

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els of *TM6SF2* and total TAG and cholesterol concentrations in the study of Kozlitina *et al.* [3], while Holmen *et al.* found a significant decrease in cholesterol but not in TAGs [14]. There is thus some variation in the association between the gene variant and changes in serum lipids as well as differences in the lipoprotein changes associated with *TM6SF2* deficiency between mouse models and between mice and man. However, it is clear that the *TM6SF2* E167K allele is not associated with hypertriglyceridemia, typically characterizing subjects with NAFLD [2].

In the present study, a comparison of the circulating TAG profiles between 'TM6SF2 NAFLD' and 'Non-risk NAFLD' showed that major circulating TAG species i.e. TAG (16:0/18:2/18:1) and TAG (16:0/18:1/18:1) were decreased. We have previously shown by analysing human liver biopsies [15] and by direct measurements of TAG fluxes across the splanchnic bed [16] that these TAGs are increased once the liver is fatty and are also the main constituents of VLDL [17]. Thus, plasma is lacking TAGs that are overrepresented in the fatty liver and in VLDL. These data are consistent with those in mice, in which silencing of *TM6SF2* decreases VLDL TAG production [3].

Regarding the lower levels of TAGs in 'PNPLA3 NAFLD' as compared to 'Non-risk NAFLD', we have previously shown [4] that absolute and relative deficiency of monounsaturated TAGs characterizes I148M variant allele carriers. Such data are in keeping with those in mice showing that overexpression of the human I148M variant enriches these TAGs in the liver [18]. In the present study, the TAG profiles of both 'TM6SF2 NAFLD' and 'PNPLA3 NAFLD' were characterized by decreases in common TAGs containing 52 carbons and two to four double bonds (Figs. 3 and 4). This could be a consequence of a decreased rate of secretion of these TAGs in VLDL [3,19]. Thus, although the *PNPLA3* and *TM6SF2* gene variants influence intrahepatic TAG handling by different mechanisms, both variants are associated with lower levels of these TAGs in the circulation.

Compared to the 'Non-risk NAFLD' group, the 'PNPLA3 NAFLD' group had lower levels of saturated and monounsaturated TAG species than the 'TM6SF2 NAFLD' group (Fig. 3). Whether this difference was due to the much lower frequency of *TM6SF2* than *PNPLA3* variant allele carriers or to differences in the function of these two proteins is unclear. Since age, male gender and obesity are each associated with an increased prevalence of NAFLD [2], one would have expected that individuals without those alleles would have differed in these aspects from the groups carrying one of the variants. Interestingly no differences in age, gender or obesity were observed. Lack of differences in the phenotypic characteristics could be due to a relatively small sample size or were perhaps due to the duration of obesity/NAFLD, which differed between the groups. On the other hand, serum TAG profiles did differ significantly and thus lack of phenotypic differences allowed comparison of circulating TAGs independent of age, gender and obesity.

Our study is the first to directly compare insulin sensitivity between *TM6SF2*^{EK/KK} and *TM6SF2*^{EE} carriers. We found that the intercepts relating liver fat and hepatic insulin sensitivity of glucose production as well as lipolysis differed significantly between the genotypes. Thus, for any given liver fat content, hepatic and peripheral insulin sensitivity were higher in *TM6SF2*^{EK/KK} as compared to *TM6SF2*^{EE} carriers (Fig. 2). Expressed in another way, liver fat content was significantly higher in *TM6SF2*^{EK/KK} as compared to *TM6SF2*^{EE} carriers for any given degree of insulin sensitivity. These data are consistent with the

lack of difference in fasting insulin concentrations in the face of different liver fat contents in the Dallas Heart Study [3]. The greater suppression of lipolysis by insulin in the *TM6SF2*^{EK/KK} than in the *TM6SF2*^{EE} carriers implies that increased FFA delivery does not contribute to increased liver fat content or VLDL TAG synthesis. Although many studies have reported a positive correlation between liver fat and hepatic insulin resistance in humans [2], many murine models accumulate liver fat without accompanying insulin resistance [20]. Previous small studies in humans with mutations or genetic variations in genes, such as *ATGL* and *CGI58*, which cause fat accumulation in the liver but not insulin resistance, support the idea that liver TAG is not sufficient to cause insulin resistance or its consequences, such as hypertriglyceridemia and dysglycemia [20].

A couple of limitations of the present study should be recognized when interpreting the results. The sample size, albeit large considering the number of lipidomic analyses was small and therefore the observed differences may underestimate true differences between the groups. The subjects were normal-weight or overweight/obese volunteers, participating in various metabolic studies who agreed to have their DNA analysed and therefore do not represent a population-based random sample. For example, the higher liver fat content in the present study in both *TM6SF2*^{EK/KK} and the *TM6SF2*^{EE} carriers (median 11.2% vs. 6.5%) compared to the European American population of the Dallas Heart Study (4.9% vs. 3.5%) could have been due to a higher BMI in the present study (33 kg/m²) than in the Dallas Heart Study cohort (~29 kg/m²) [3].

After submission of this manuscript, Liu *et al.* reported that the *TM6SF2* variant, encoding E167K is associated with hepatic fibrosis/cirrhosis, independent of age, BMI, type 2 diabetes mellitus and *PNPLA3* rs738409 genotype [21]. The association of the *TM6SF2* variant with steatosis was marginally significant. We found the amount of liver fat to be clearly dependent on the *TM6SF2* genotype, perhaps because use of ¹H-MRS enables measurement of liver fat in a larger volume than when using a liver biopsy. Our data do not exclude the possibility that the gene variant is associated with fibrosis since liver biopsies were not obtained.

We conclude that NAFLD, associated with the *TM6SF2* variant encoding E167K, is metabolically silent. It increases liver fat content but this is not accompanied by a decrease in whole body or hepatic or adipose tissue insulin sensitivity. It is associated with decreases of circulating total TAGs and those that are enriched in the liver and VLDL of subjects with 'Non-risk NAFLD'. Lack of these metabolic sequelae could decrease the risk for type 2 diabetes and cardiovascular diseases in these NAFLD subjects. Genotyping patients to identify carriers of the *PNPLA3* and *TM6SF2* variants might thus help in the identification of subjects who are at risk of developing advanced liver disease but not metabolic problems associated with 'Non-risk NAFLD'.

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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.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2014.10.010>.

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