Effects of TM6SF2 E167K on hepatic lipid and very low-density lipoprotein metabolism in humans

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Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic lipid accumulation. The transmembrane 6 superfamily member 2 (TM6SF2) E167K genetic variant associates with NAFLD and with reduced plasma triglyceride levels in humans. However, the molecular mechanisms underlying these associations remain unclear. We hypothesized that TM6SF2 E167K affects hepatic very low-density lipoprotein (VLDL) secretion and studied the kinetics of apolipoprotein B100 (apoB100) and triglyceride metabolism in VLDL in homozygous subjects. In 10 homozygote TM6SF2 E167K carriers and 10 matched controls, we employed stable-isotope tracer and compartmental modeling techniques to determine apoB100 and triglyceride kinetics in the 2 major VLDL subfractions: large triglyceride-rich VLDL, and smaller, less triglyceride-rich VLDL. VLDL-apoB100 production was markedly reduced in homozygote TM6SF2 E167K carriers compared with controls. Likewise, VLDL-triglyceride production was 35% lower in the TM6SF2 E167K carriers. In contrast, the direct production rates for VLDL-apoB100 and triglyceride were not different between carriers and controls. In conclusion, the TM6SF2 E167K genetic variant was linked to a specific reduction in hepatic secretion of large triglyceride-rich VLDL. The impaired secretion of VLDL explains the reduced plasma triglyceride concentration and provides a basis for understanding the lower risk of cardiovascular disease associated with the TM6SF2 E167K genetic variant.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a condition defined by excessive (>5% by weight) fat accumulation in the liver. As a consequence of the obesity and diabetes epidemic, NAFLD has become the most common liver disorder in developed countries, with more than 100 million adults and children affected in the United States alone (1). Although NAFLD may progress to severe liver disease, including nonalcoholic steatohepatitis and cirrhosis (2, 3), the most common cause of death in patients with the condition is atherosclerotic cardiovascular disease (4).

NAFLD has a multifactorial etiology that includes a strong genetic component. The transmembrane 6 superfamily member 2 (TM6SF2) rs58542926 variant, encoding for a glutamic acid to lysine substitution at position 167 of the amino acid sequence (E167K), has been identified as one of the most important genetic determinants of hepatic fat content (5–7). This single nucleotide variant reduces expression of the TM6SF2 protein by 46% in liver cells (7), and its prevalence is around 7% in Caucasian populations (8). Despite extensive investigation, the molecular function of TM6SF2 remains unclear. Localized on chromosome 19, the gene is expressed mainly in the liver, small intestine, and kidney (7). It encodes a 351–amino acid
apoB100, which is reflective of the number of VLDL 1 particles secreted per day, was markedly reduced and VLDL2 TG, the relative abundance of unsaturated fatty acids in the a 50% reduction of the amount of this lipid in VLDL 1 (Table 1). carriers compared with controls (Table 1). The reduction in plasma TG was mainly attributable to TM6SF2 = 0.011) were significantly lower in homozygote = 0.003) and plasma TG (1.0 ± 0.5 vs. 1.4 ± 0.3 mmol/L; = 0.023) (Table 1). Likewise, production of VLDL 1-TG was 35% lower in the former compared with the...
latter group (21,307 ± 10,831 vs. 32,774 ± 13,055 mg/dL, P = 0.043). In contrast, the direct production rates for VLDL-2-apoB100 and -TG were not different between carriers and noncarriers (Table 3). Fractional catabolic rates (FCRs) for VLDL-1 and VLDL-2-apoB100 and -TG did not differ significantly between the groups (Table 3). There were no significant differences in hepatic DNL measured in VLDL1 or plasma β-hydroxybutyrate levels (i.e., a marker of hepatic β-oxidation) between the 2 groups of study subjects (Table 1).

Because plasma apoC-III levels are strong predictors of VLDL1-TG FCR (24) and there was a tendency to lower levels of this apoprotein in the TM6SF2 homozygotes, we explored the association of plasma apoC-III levels with VLDL1-TG FCR in the 2 subject groups and found that the difference in apoC-III plasma levels did not correlate with the difference in VLDL1-FCR (r = –0.21, P = 0.39).  

Discussion
The aim of the present study was to elucidate further the phenotype associated with the genetic variant TM6SF2 E167K, in particular how it affects hepatic VLDL secretion, and by extrapolation risk of atherosclerotic cardiovascular disease. Our results show clearly that in subjects homozygous for this variant, there is a specific impact on the synthesis and secretion of large TG-rich VLDL1 but no effect on the direct secretion of the smaller, less TG-rich VLDL2. This finding is informative since we know from previous studies that production of these 2 VLDL subfractions is regulated independently (25). There was no significant influence of the TM6SF2 E167K variant on the lipolysis or clearance rates of either

| Table 1. Baseline characteristics of study subjects |
|-----------------------------------------------|
| CONTROL | TM6SF2 | Fold | P value |
| Age (y)  | 54.9 ± 9 | 58.4 ± 12.5 | 1.06 | 0.21 |
| Syst. BP (mmHg) | 134 ± 11 | 138 ± 16 | 1.02 | 0.68 |
| Diast. BP (mmHg) | 83.1 ± 5.9 | 85 ± 6.4 | 1.02 | 0.76 |
| BMI (kg/m²) | 27.6 ± 3.1 | 27.5 ± 2.7 | 1.00 | 1.00 |
| Body weight (kg) | 83.9 ± 10.2 | 77.9 ± 10.2 | 0.93 | 0.11 |
| Waist (cm) | 98.6 ± 8.5 | 99.8 ± 7.2 | 1.01 | 0.85 |
| SAT area (cm²) | 3324 ± 742 | 3642 ± 904 | 1.10 | 0.35 |
| VAT area (cm²) | 1898 ± 923 | 1431 ± 311 | 0.75 | 0.35 |
| Liver fat (%) | 4.0 ± 5.2 | 8.4 ± 6.1 | 2.10 | 0.023 |
| Glucose (mmol/L) | 5.4 ± 0.5 | 5.7 ± 0.4 | 1.06 | 0.24 |
| Insulin (mU/L) | 8.1 ± 6.2 | 11.3 ± 5.9 | 1.40 | 0.15 |
| HOMA-IR | 2.0 ± 1.5 | 2.9 ± 1.6 | 1.45 | 0.19 |
| ALT (U/L) | 28 ± 16 | 28 ± 10 | 0.99 | 0.57 |
| ALP (U/L) | 67 ± 10 | 65 ± 24 | 0.98 | 0.44 |
| AST (U/L) | 31 ± 9 | 27 ± 5 | 0.87 | 0.38 |
| Total chol (mmol/L) | 5.1 ± 0.5 | 4.8 ± 0.9 | 0.94 | 0.25 |
| LDL-chol (mmol/L) | 3.4 ± 0.5 | 3.0 ± 0.7 | 0.88 | 0.12 |
| HDL-chol (mmol/L) | 1.2 ± 0.4 | 1.6 ± 0.5 | 1.33 | 0.054 |
| Fasting TG (mmol/L) | 1.4 ± 0.3 | 1.0 ± 0.5 | 0.71 | 0.011 |
| VLDL1-TG (mmol/L) | 0.6 ± 0.3 | 0.3 ± 0.2 | 0.50 | 0.011 |
| VLDL2-TG (mmol/L) | 0.2 ± 0.1 | 0.2 ± 0.1 | 1.00 | 0.089 |
| ApoB (mg/dL) | 106 ± 19 | 81 ± 19 | 0.76 | 0.003 |
| VLDL1-apoB (mg/dL) | 2.0 ± 1.0 | 1.0 ± 0.6 | 0.50 | 0.029 |
| VLDL2-apoB (mg/dL) | 3.0 ± 1.6 | 2.1 ± 1.1 | 0.70 | 0.22 |
| ApoC-III (mg/dL) | 12.1 ± 2.5 | 9.9 ± 1.9 | 0.82 | 0.064 |
| FFA (μmol/L) | 428 ± 149 | 534 ± 223 | 1.25 | 0.28 |
| DNL in VLDL1 (%) | 5.9 ± 5.1 | 8.6 ± 10.1 | 1.46 | 1.00 |
| β-OH butyrate (mg/dL) | 0.9 ± 0.6 | 1.2 ± 0.6 | 1.33 | 0.14 |

Fold difference is the ratio of means in the groups, >1 is higher in the TM6SF2 homozygote group. P values are from the Kruskal-Wallis test. Bold text indicates P < 0.05. Syst., systolic; diast., diastolic; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; FFA, free fatty acids; DNL, de novo lipogenesis; β-OH butyrate, β-hydroxybutyrate.
VLDL subfraction. The decrease in VLDL subfraction production is the likely proximal cause of the reduction in plasma apoB, although it should be noted that the decrease in VLDL apoB concentration in TM6SF2 E167K homozygotes was insufficient to account for the much larger decrease in apoB overall. It is likely that a consequent fall in the levels of the delipidation products of VLDL — remnants and LDL — contributed to the lower total apoB (25) and also to the reduced risk of atherosclerotic cardiovascular disease associated with this genetic variant (20, 26).

Cell culture studies have revealed that the assembly of VLDL in hepatocytes is a multistage process (27). In the first step, primordial pre-VLDL particles are formed in the ER. These then enter the secretory pathway and are converted to VLDL-sized particles by incremental addition of TG in a stepwise lipidation process (28). The precursor VLDL particles either are released from the cell (and are detected as “direct” VLDL apoB secretion in Table 3) or undergo a further lipidation step to become large, TG-rich VLDL, which are then secreted (28, 29). The conversion of VLDL-sized precursor particles to VLDL occurs in a smooth membrane compartment, likely the ERGIC (30). The process requires bulk addition of TG because VLDL contains a mean of 43,154 molecules of TG per particle (i.e., per apoB, Table 2) compared with the 11,180 per particle in VLDL. Formation of VLDL is believed to be mediated by fusion of a lipid droplet formed in the smooth ER with the VLDL-sized precursor (31).

Thus, assembly of TG-rich VLDL is highly dependent on the availability of TGs to form these smooth ER lipid droplets, which in turn requires a ready supply of TG within the hepatocyte (32). This can come from DNL, fatty acid uptake from the circulation, or mobilization of cytoplasmic lipid stores (macroporous lipid droplets) (33). The last is a major source, and VLDL secretion is a way in which the liver can regulate the amount of stored intracellular TG (33, 34). In most subject groups it is possible to observe an association between the amount of TG in intracellular stores (liver fat) and the secretion rates of VLDL apoB100 and TG (34). Inheritance of the TM6SF2 E167K variant disrupts this relationship; liver fat increases but VLDL secretion is reduced.

| Table 2. Lipid class composition (molecules per apoB) of VLDL<sub>1</sub> and VLDL<sub>2</sub> in control and homozygous TM6SF2 carriers |
|---------------------------------------------------------------|
| CONTROL | TM6SF2 | Fold | P value |
| CE | 15,754 ± 6899 | 16,615 ± 4946 | 1.05 | 0.85 |
| LPC | 621 ± 376 | 1007 ± 476 | 1.62 | 0.08 |
| PC | 11,870 ± 5557 | 11,657 ± 3598 | 0.98 | 0.68 |
| PE | 659 ± 209 | 891 ± 392 | 1.35 | 0.17 |
| SM | 2155 ± 1024 | 2561 ± 949 | 1.19 | 0.31 |
| TG | 43,154 ± 16,509 | 50,537 ± 14,357 | 1.17 | 0.11 |
| CE | 13,207 ± 4479 | 10,410 ± 6274 | 0.79 | 0.28 |
| LPC | 289 ± 170 | 359 ± 247 | 1.24 | 0.53 |
| PC | 4795 ± 1634 | 3616 ± 2506 | 0.75 | 0.14 |
| PE | 124 ± 53 | 131 ± 113 | 1.06 | 0.68 |
| SM | 1888 ± 845 | 1329 ± 708 | 0.7 | 0.14 |
| TG | 11,180 ± 4013 | 9078 ± 5425 | 0.81 | 0.35 |

Data are reported as mean ± SD and P values are calculated using the Kruskal-Wallis test. CE, cholesteryl esters; LPC, lysophosphatidylcholines; PC, phosphatidylcholines; PE, phosphatidylethanolamines; SM, sphingomyelins, TG, triglycerides.
expansion or by fusion with other droplets (33, 36, 37). The availability of phospholipids, especially PC, is considered an important factor in facilitating the increase in size of lipid droplets (38). Krahmer et al. demonstrated that expanding lipid droplets recruit CTP:phosphocholine cytidylyltransferase, the rate-limiting enzyme for PC synthesis, and that this enzyme is activated when it binds to the surface of the droplets (38). Luukkonen et al. demonstrated that the TM6SF2 E167K genetic variant leads to altered hepatic lipid metabolism, resulting in relative PC deficiency due to impaired synthesis of this lipid from polyunsaturated fatty acids (22). In accord with this finding, we observed significant decrease in the relative abundance of longer and more unsaturated fatty acids in VLDL1 and VLDL2 TGs in homozygous carriers of the TM6SF2 E167K genetic variant. Thus, it is possible that PC deficiency due to inheritance of the TM6SF2 E167K variant leads to an assembly defect and failure to synthesize and secrete large TG-rich VLDL particles from the liver. However, the observation about fatty acid length and saturation was observed in both large TG-rich VLDL1 and smaller VLDL2 fractions. This finding argues against a specific effect on the assembly of large VLDL1 particles.

It is also possible that the impact of the genetic variant is secondary and caused by other genes that are coexpressing with TM6SF2 as proposed by Luukkonen et al. (22). This action has been suggested to explain the reduced hepatic expression of apoC-III in subjects with the TM6SF2 E167K variant (22). ApoC-III has a number of roles in VLDL metabolism. Pertinent to the present discussion is the proposal that this apolipoprotein may exert its effects on lipid substrate utilization for the second-step VLDL assembly process (39). Very recently, Li et al. reported that TM6SF2 stabilizes apoB by interacting with ER lipid raft protein 1 and 2, which mediates the ER-associated degradation of inositol 1,4,5-trisphosphate receptors (40). However, the relevance of these findings for human pathophysiology is still unclear, and it is not evident how defective apoB stabilization would specifically impair hepatic VLDL1 secretion.

In conclusion, here we demonstrate that the TM6SF2 E167K genetic variant is linked to a specific reduction in hepatic secretion of VLDL1. The proposed underlying mechanism is a relative failure to secrete large, TG-rich lipoproteins, and the inability to export lipid efficiently through the VLDL pathway is a satisfactory explanation for the accumulation of liver fat. The 35% reduction in flow of apoB down the delipidation cascade will have predictable consequences for the products of lipolysis of VLDL1 — remnant VLDL particles and LDL — which are implicated as causal factors in atherosclerosis (25). These metabolic perturbations explain the reduced plasma TG concentrations (7, 26, 41), and lower risk of cardiovascular disease (20, 26) associated with the TM6SF2 E167K genetic variant.
Methods

Subjects. A group of 20 subjects, recruited on the basis of their TM6SF2 E167K and PNPLA3 I143M genotypes, participated in this study. Twelve subjects, identified from the THL Biobank (a country-wide biobank that collects and stores research samples from all over Finland, https://thl.fi/en/web/thl-biobank/about-thl-biobank), had participated in earlier studies where exome sequencing or genotyping and imputation had been performed to explore genes involved in lipid metabolism. The remaining 8 subjects came from previously examined kinetic study cohorts (24, 42, 43). All had given oral consent that allowed them to be invited to further studies focused on lipid metabolism. Two groups were included in this study: 10 homozygote carriers for TM6SF2 E167K (9 men and 1 woman) and 10 control subjects (9 men and 1 woman), matched for degree of obesity and glycemic status, who were not carriers of TM6SF2 E167K and also were not homozygous for PNPLA3 I143M (Table 1). Inclusion criteria were age 18–70 years, nonsmoking status, and BMI < 33 kg/m². Exclusion criteria included a history of cardiovascular or other severe disease, any condition affecting lipid levels, abnormalities in thyroid or kidney function, liver disease other than NAFLD, abnormal blood count, glycated hemoglobin (HbA1c) > 42 mmol/mol, LDL-cholesterol > 4.5 mmol/L, plasma TG > 2.5 mmol/L, uncontrolled hypertension (>160 mmHg systolic and /or >105 mmHg diastolic blood pressure), use of thiazide diuretics (>25 mg/d), use of medications affecting lipid or glucose metabolism, or high alcohol consumption (>30 g/d for men and >20 g for women). The participants were asked to abstain from alcohol and strenuous physical exercise during leisure time before metabolic investigations.

Study design. The kinetic studies were performed as described previously (24, 44). On the evening before the VLDL apoB100/TG kinetic study, ²H₂O (2 g/kg) was given between 1800 and 2200 hours to give an assessment of the degree of the contribution of hepatic DNL to VLDL TGs (see refs. 45, 46). The subjects came back at 7:30 am to the research unit of the Helsinki University Hospital after a 12-hour overnight fast. An indwelling cannula was inserted into an antecubital vein for blood sampling, and a second cannula was inserted into the opposite antecubital vein. The subjects received a bolus injection of [²H₅]glycerol (500 mg) and [²H₃]leucine (7 mg/kg). Blood samples were drawn before tracer injection and at frequent intervals thereafter (24, 44).

Lipoprotein isolation. VLDL₁ and VLDL₂ were separated from plasma as described previously (44). ApoB100 was obtained from the 2 VLDL subfractions and hydrolyzed, derivatized, and subjected to gas chromatography mass spectrometry to measure tracer leucine enrichment (44). Likewise, TG was extracted from the VLDL fractions and the tracer glycerol enrichment determined (44). The particle composition and apoB mass of VLDL₁ and VLDL₂ were determined before and at 4 and 8 hours after tracer injection. The subjects continued to fast until 5 pm, when the last blood sample was taken and they were served a standard dinner.

Biochemical analyses. Fasting TG and cholesterol concentrations in total plasma and in the VLDL₁ and VLDL₂ fractions, and HDL-cholesterol and LDL-cholesterol, were analyzed by automated enzymatic methods using the Konelab 60i analyzer (Thermo Fisher Scientific). Concentrations of glucose (Gluco-quant, Roche Diagnostics) and insulin (electrochemiluminescence with Roche sandwich immunoassay using
Cobas autoanalyzer) were measured in fasting blood samples and HOMA-IR was calculated (47). Plasma levels of apoC-III were measured immune-turbidometrically (Kamiya Biochemical Company) and β-hydroxybutyrate concentrations by using a β-hydroxybutyrate FS kit (Diagnostic Systems) on a Konelab 60i analyzer (Thermo Fisher Scientific). Plasma fatty acids were analyzed using an automatic enzymatic colorimetric method (Wako Chemicals).

**Lipidomics.** Lipid extraction of 50 μL of each VLDL fraction was performed using the BUME method (48). TGs were quantified by direct infusion (shotgun) analysis on a QTRAP 5500 mass spectrometer (SCI-EX) equipped with a robotic nanoflow ion source, TriVersa NanoMate (Advion BioSciences). The analysis was performed in positive ion mode by neutral loss detection of 10 common acyl fragments formed during collision-induced dissociation according to previous work (49). Glyceryl-1,2,3-trioleatoate (CDN Isotopes) was added during the extraction and used for quantification.

**Imaging.** Liver fat content was measured using proton magnetic spectroscopy (1.5 T whole-body device) (34), and subcutaneous and visceral fat were measured by magnetic resonance imaging (50). All imaging analyses were performed by a single person. Subjects were advised to fast for 4 hours before scans were taken.

**Genotyping.** The genotypes of participants were confirmed upon recruitment to the present study. DNA was extracted from blood using DNeasy Blood & Tissue Kit (Qiagen) and used to determine TM6SF2 rs58542926 (E167K) and PNPLA3 rs738409 (I148M) status by TaqMan assays (Life Technologies, Thermo Fisher Scientific) using CFX384 Real Time PCR detection system (Bio-Rad Laboratories). Data analysis was performed using Bio-Rad CFX manager software.

**Modeling.** The multicompartmental model used to analyze simultaneously VLDL apoB100 and TG kinetics was constructed using SAAM II (The Epsilon Group) (51). Inputs to the model were (a) injected amounts of [3H]-leucine and [2H5] glycerol, (b) apoB100 and TG pool sizes in VLDL1 and VLDL2, (c) enrichment curve of plasma leucine, (d) enrichment of deuterated leucine in in VLDL1- and VLDL2-apoB100, and (e) enrichment of deuterated glycerol in VLDL1- and VLDL2-TG (44). Outputs were production (in mg/d) and fractional clearance rates (in pools/d) for apoB100 and TG for each subfraction and the transfer rates from VLDL1 to VLDL2.

**Statistics.** Statistical calculations were performed using R (version 3.6.3). Data are reported as mean ± SD, and values were calculated using the Kruskal-Wallis test. Correlation coefficients refer to the Spearman’s rank correlation coefficient. Trend analyses for lipidomics data were performed using linear mixed models with number of double bonds as fixed effect and subject as random effect. Data were normalized to mean of control group; P values correspond to tests of the linear effect of double bonds differing from 0. P < 0.05 was considered statistically significant.

**Study approval.** The study protocol was approved by the Ethics Committee of Helsinki University Central Hospital, Helsinki, Finland. ClinicalTrials.gov Identifier: NCT04209816. The study was performed in accordance with the Declaration of Helsinki and the European Medicines Agency Note for guidance on good clinical practice. All study participants signed a written informed consent form before any study procedures were initiated.

**Author contributions**
The authors contributed to the present work as follows: JB, NM, and MRT contributed to conception and design; MA, EB, NM, SS, JR, MS, PR, S Ripatti, AP, RMM, AH, and S Romeo to the acquisition of data or analysis; and JB, MA, EB, CJP, and MRT to the interpretation of data. JB, MA, CJP, and MRT drafted the original and revised manuscripts, and all authors approved the final version to be published.

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