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

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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
HbA_{1c}	glycosylated hemoglobin 1c
HDL	high density lipoprotein
^{1}H-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

ABSTRACT

Aims: The Glu167Lys (E167K) variant in *TM6SF2* was recently shown to influence liver fat (LFAT) content. We aimed at studying how the variant influences the circulating triacylglycerol (TAG) signature and whether it influences hepatic or adipose tissue insulin sensitivity.

Methods: We genotyped 300 Finnish subjects for E167K (rs58542926) in *TM6SF2* and for I148M (rs738409) in *PNPLA3* in whom LFAT was measured using ^{1}H-MRS and circulating lipids by UPLC-MS. We compared plasma lipidome between E167K carriers ($TM6SF2^{EK/KK}$) and non-carriers ($TM6SF2^{EE}$), and between three groups of NAFLD: i) carriers of E167K but not the I148M variant in PNPLA3 ('TM6SF2 NAFLD'), ii) carriers of the I148M but not the E167K variant ('PNPLA3 NAFLD') and iii) non-carriers of either risk allele ('Non-risk NAFLD'). Hepatic and adipose tissue insulin sensitivities were measured using the euglycemic hyperinsulinemic clamp technique combined with infusion of [3-^{3}H]glucose in 111 subjects.

Results: LFAT content was 34% higher in $TM6SF2^{EK/KK}$ (13.07±1.57%) than $TM6SF2^{EE}$ (9.77±0.58%, $p = 0.013$). Insulin sensitivities of glucose production and lipolysis were significantly higher at any given LFAT in the $TM6SF2^{EK/KK}$ than in the $TM6SF2^{EE}$ group. Comparison of 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 distinct subtype of NAFLD characterized by preserved insulin sensitivity of lipolysis and hepatic glucose production and lack of hypertriglyceridemia despite clearly increased LFAT content.

Introduction

Genetic factors contribute to the pathogenesis of non-alcoholic fatty liver disease (NAFLD). In 2008, an allele in *PNPLA3* (rs738409[G], encoding I148M) was found to be highly significantly associated with NAFLD in three different ethnic group [1]. This finding has subsequently been robustly replicated in over 50 studies including eight genome wide association scans [2]. Recently, genetic variation in *TM6SF2* at rs58542926 was shown to confer susceptibility to NAFLD independent of genetic variation at rs738409 in *PNPLA3* 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 variant allele was higher in individuals of European (7.2%, n=882) than of other ancestry groups. *TM6SF2* variant allele carriers had significantly lower 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 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 permitting blood sampling and analysis of polymorphisms in genes related to LFAT content.

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

Lipidomic analysis with UPLC-MS

An unthawed plasma sample was extracted for lipidomic analysis (*vide infra*). An established platform based on Acquity Ultra Performance LC™ coupled to quadruple time-of-flight mass spectrometry (UPLC-MS) was used to analyze 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 labeled PC (16:1/0:0-D_3), PC(16:1/16:1-D_6) and TAG(16:0/16:0/16:0-^{13}C3) was added.

The extracts were analyzed on a Waters Q-Tof Premier mass spectrometer combined with an Acquity Ultra Performance LC™. The column (at 50 °C) was an Acquity UPLC™ BEH C18 2.1 × 100 mm with 1.7 µm particles. The solvent system included A. ultrapure water (1% 1 M 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 sec.

The data processing including alignment of peaks, peak integration, normalization and identification was done by using MZmine 2 software [6] and the 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 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, PC(17:0/17:0) for a retention time between 300 s and 410 s, and TAG(17:0/17:0/17:0) for longer retention times. For further identification of unknown lipids, please see supplementary material for detail.

LFAT content

LFAT content was measured by proton magnetic resonance spectroscopy (^{1}H-MRS) as previously described and validated against histologic measurement of LFAT [7]. In a few subjects (n=36), LFAT was measured using a liver biopsy. The fat content of the liver biopsy specimens (the percentage of hepatocytes with macrovesicular steatosis) was determined using hematoxylin-eosin staining and converted to ^{1}H-MRS LFAT percentage units, as previously described [7]. NAFLD was defined as LFAT ≥55.6 mg triglyceride per gram of liver tissue or ≥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 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 of the antilipolytic effect of insulin were assessed by the euglycemic hyperinsulinemic clamp technique combined with infusion of [3-^{3}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 % suppression of hepatic glucose production by the mean S-insulin concentration (mU/l). Adipose tissue insulin sensitivity was calculated by dividing the % suppression of S-free fatty acids (S-FFA) by insulin by the mean S-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 spina iliaca superior and the lower rib margin. Body height was recorded to the nearest 0.5 centimeter. Blood pressure, fP-glucose, fS-insulin, fS-LDL cholesterol, total serum cholesterol, fS-HDL cholesterol, fS-TAG, AST, ALT, ALP and GGT concentrations were measured as previously described [10]. Homeostasis model assessment of insulin resistance (HOMA-IR) was estimated by using formula: HOMA-IR = fS-insulin (mU/l) × fP-glucose (mmol/l)/22.5 [11]. '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 means ± SEM and non-normally distributed data are shown as medians 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 TM6SF2 variant and wide-type by using student's t-tests. Multiple comparisons were corrected by using Benjamini-Hochberg's method [13]. The comparisons of abundances of TAG molecules were illustrated by heatmaps, which show fatty acid chain lengths and number of double bonds. Log2 transformed ratios of the TAG values of 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±SE: 13.07±1.57% *vs* 9.77±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±SE: 19.4±3.0 vs. 13.4±1.1%, p = 0.02) and were almost significantly less obese (32.0±1.7 vs 36.3±1.0 kg/m^2, p = 0.059). The groups were comparable with respect to age (54±3 vs. 52±1 yrs), gender (80% vs. 60% men, p = 0.37), fasting serum TAGs (2.1±0.3 vs. 2.2±0.2 mmol/l), insulin (14.1±1.7 vs. 16.4±1.3 mU/l), HOMA-IR (6.7±1.0 vs. 6.0±0.5) and *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 fS-TAGs measured enzymatically in the clinical laboratory (1.81±0.077

mmol/l, n=300) were highly correlated with the sum of plasma TAGs identified by UPLC-MS (0.83±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 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 *TM6SF2* genotype and insulin sensitivity of 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 the 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**; **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 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 extend previous data by examining how the variant (and increased liver fat) influences the circulating TAG profile and directly measured insulin sensitivity. We also analyzed 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 the *PNPLA3* I148M variant allele ('TM6SF2 NAFLD'), NAFLD associated with the *PNPLA3* but not the *TM6SF2* E167K ('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 of 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 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 ^{1}H-MRS technique was 34% increased in carriers of the E167K minor allele in the present study.

As in the Dallas Heart Study in the 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, selective knockdown of *TM6SF2* in the liver by short hairpin RNAs decreased protein levels 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 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 mouse and man. However, it is clear that the *TM6SF2* E167K allele is not associated with the hypertriglyceridemia typically characterizing subjects with NAFLD [2].

In the present study, 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 analyzing human liver biopsies [15] and by direct measurement 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, which 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 (**Fig. 3** and **Fig. 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 aging, male gender and obesity are each associated with an increased prevalence of NAFLD [2], one would have expected that individuals without the alleles would have differed in these respects 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 relatively small sample size or perhaps duration of obesity/NAFLD 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 a 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 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 analyzes 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 analyzed 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 Americans in the population-based Dallas Heart Study (4.9 vs. 3.5%) could have been due a higher BMI in the present study (33 kg/m^2) than the Dallas Heart Study cohort (~29 kg/m^2) [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 ^{1}H-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 the NAFLD associated with the *TM6SF2* variant encoding E167K is metabolically silent. It increases liver fat content but this is not accompanied by 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 from type 2 diabetes and cardiovascular disease in these NAFLD subjects. Genotyping to identify carriers of the *PNPLA3* and *TM6SF2* variants might thus help in identification of subjects who are at risk of developing advanced liver disease but not the metabolic problems associated with 'Non-risk NAFLD'.

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

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

Data are in n (%), means ± SEM or median (25^{th}-75^{th} percentile), as appropriate.

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

Total	TM6SF2 NAFLD (n=13)	PNPLA3 NAFLD (n=77)	Non-risk NAFLD (n=64)
Age (years)	49.5±13.3	47.5±11.2	47.6±10.7
Gender (% women)	46	51	53
Type 2 Diabetes (%)	38	45	33
BMI (kg/m^2)	32.6±6.1	35.7±7.8	33.5±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±18	135±16	134±15
Diastolic BP (mmHg)	86±11	85±9	83±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 (µmol/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 (%), means ± SEM or median (25^{th}-75^{th} percentile). $^{\dagger}p < 0.05$ for one-way ANOVA; * $p < 0.05$ for LSD post hoc test compared with the 'Non-NAFLD risk'. #: 'NASH score' estimated based on AST, *PNPLA3* genotype and fS-insulin [12].

FIGURE LEGENDS

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

Fig. 2. Relationships between liver fat and hepatic (*top panel*) and adipose tissue insulin sensitivity (*bottom panel*) in $TM6SF2^{EK/KK}$ (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.

Fig. 3. Comparison of concentrations of plasma TAGs 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 (*$p < 0.05$, **$p < 0.01$).

Fig. 4. Distribution of differences in plasma TAG species between 'TM6SF2 NAFLD', PNPLA3 NAFLD' and 'Non-risk NAFLD' groups. 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 (*$p < 0.05$, **$p < 0.01$).

Figure 1

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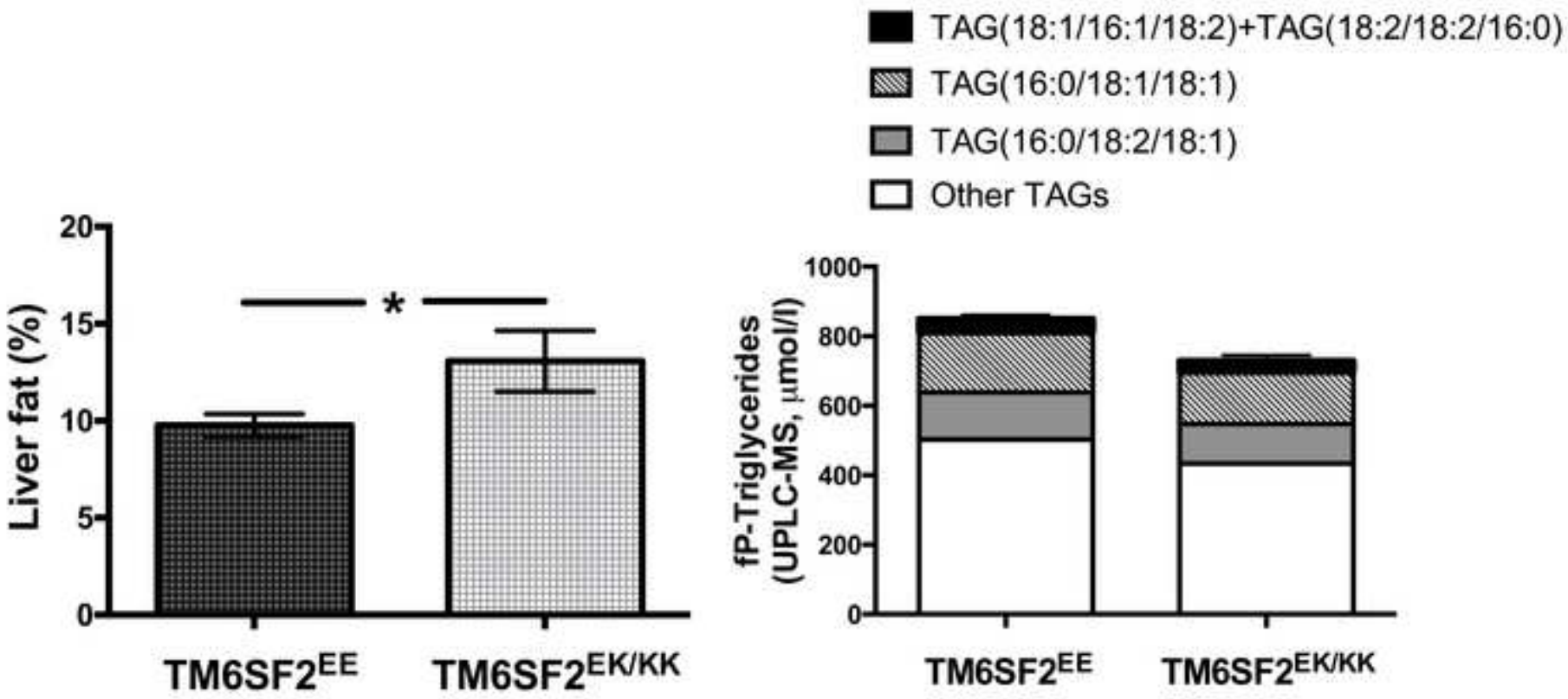

Figure 2

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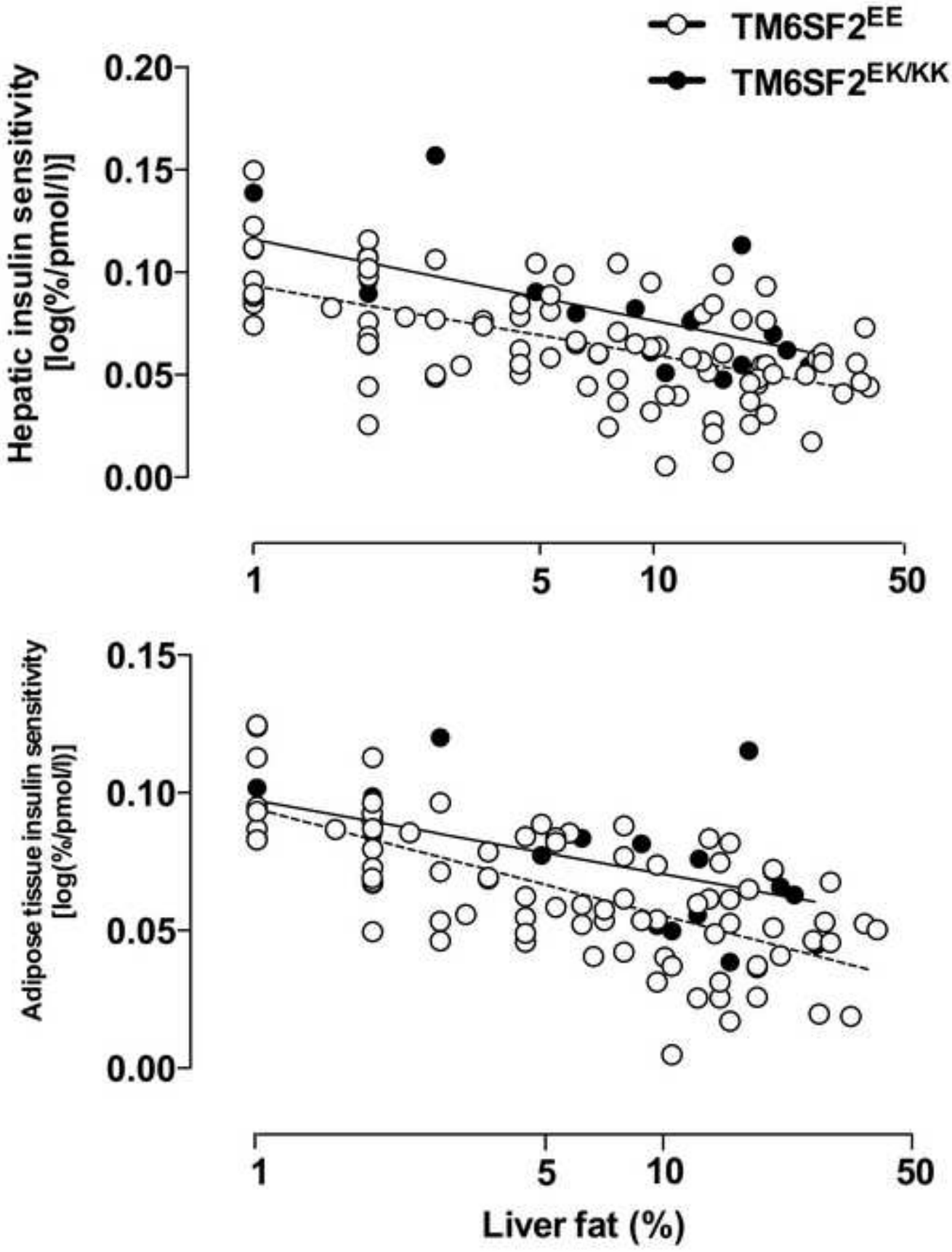

Figure 3

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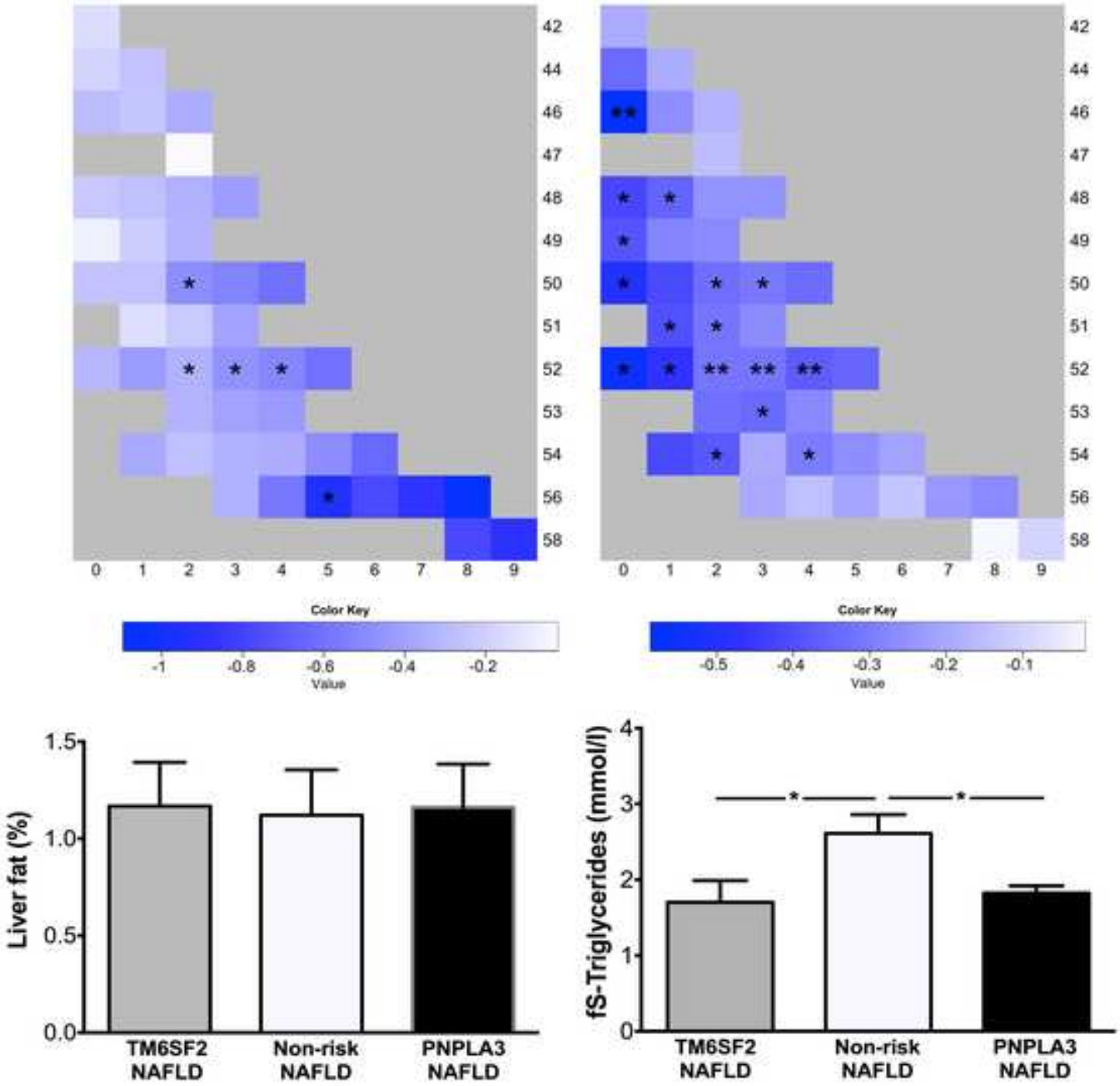

Figure 4

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