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TM6SF2 rs58542926 variant affects postprandial lipoprotein metabolism and glucose homeostasis in NAFLD.

A clue to the opposite impact of TM6SF2 variant on liver and cardio-metabolic disease?

RUNNING TITLE: *TM6SF2* POLYMORPHISM IN NAFLD

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Tables 4 Figures 1

ABBREVIATIONS: AI: adaptation index; AUC: area under the curve; BIA: bioelectrical impedance analysis; BMI: body mass index; CGI: CP-genic index; Chol: cholesterol; CK-18: cytokeratin-18; CVD: cardiovascular disease; DI: disposition index; EPIC: European Prospective Investigation into Cancer and Nutrition; FFA: free fatty acids; FIVGTT: frequently-sampled intravenous glucose tolerance test; HII: hepatic iron index; IGI: insulinogenic index; IAUC: incremental AUC; LIC: liver iron concentration; NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; NT: nitrotyrosine OFTT: oral fat load test; OGIS: oral glucose insulin sensitivity index; OGTT: oral glucose tolerance test; oxLDL: oxidized LDLs; SNP: single nucleotide polymorphism; TAS: total antioxidant status; Tg: triglyceride; TNF: tumor necrosis factor; TRLP: triglyceride-rich lipoproteins; VLDL: very low density lipoprotein

ABSTRACT.

Mechanisms underlying the opposite effects of TM6SF2 rs58542926 C>T polymorphism on liver injury and cardio-metabolic risk in NAFLD are unclear. We assessed the impact of this polymorphism on postprandial lipoprotein metabolism, glucose homeostasis, and nutrient oxidation in NAFLD.

Sixty nonobese, nondiabetic, normolipidemic biopsy-proven NAFLD patients and 60 matched controls genotyped for TM6SF2 C>T polymorphism underwent: indirect calorimetry, an oral fat tolerance test with measurement of plasma lipoprotein subfractions, adipokines, incretin GIP, and an OGTT with Minimal Model analysis of glucose homeostasis.

TM6SF2 T-allele was associated with higher hepatic and adipose insulin resistance, with impaired pancreatic ß-cell function and incretin effect and with higher muscle insulin sensitivity and whole-body fat oxidation rate.

Compared with TM6SF2 C-allele, T-allele entailed lower postprandial lipemia and nefaemia, a less atherogenic lipoprotein profile, a postprandial cholesterol redistribution from smaller, atherogenic lipoprotein subfractions to larger intestinal and hepatic VLDL1 subfration. Postprandial plasma VLDL1-cholesterol response independently predicted the severity of liver histology.

In conclusion, TM6SF2 C>T polymorphism affects nutrient oxidation, glucose homeostasis, and postprandial lipoprotein, adipokine and GIP responses to fat ingestion, independently of fasting values. These differences may contribute to the dual and opposite effect of this polymorphism on liver injury and cardio-metabolic risk in NAFLD.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) confers an increased risk of liver-related complications (largely limited to its progressive form, non-alcoholic steatohepatitis, NASH), of type 2 diabetes (T2DM), and of cardiovascular disease (CVD)[1,2]. The wide inter-individual variability in the risk of hepatic and extra-hepatic complications in NAFLD may reflect the interplay between genetic and environmental factors. While in the general population an association between the type and amount of dietary fat and the development of obesity, CVD and T2DM has been demonstrated [3],data linking dietary fat to the presence and severity of NAFLD are controversial [4,5].We hypothesized a genetically determined susceptibility to dietary fat lipotoxicity modulates liver injury and cardio-metabolic risk in NAFLD.

The single nucleotide polymorphism (SNP) rs58542926 C>T in the *Transmembrane 6 superfamily member 2* gene (*TM6SF2*) has been recently linked to the severity of NAFLD in genome-wide association studies (GWAS) [6,**7**]: TM6SF2 T-allele, encoding the E167K aminoacidic substitution, results in reduced transcript levels of its product protein, which is expressed in humans in the liver, intestine, adipose tissue and pancreatic β-cells and has unclear biological function[8,9].

TM6SF2 C>T variant has been linked to a reduced LDL-cholesterol level and cardiovascular risk and to an increased risk of type 2 diabetes [10,11]. Mechanisms connecting TM6SF2 C>T polymorphism to liver injury and cardio-metabolic risk are unclear. The impaired hepatic VLDL secretion associated with TM6SF2 T-allele[8,9] may not be the main mechanism mediating NASH, as enhanced lipid storage into neutral triglycerides protects against liver injury[12]. Furthermore, the reduced CVD risk associated with TM6SF2 T-allele is not fully explained by lower fasting cholesterol levels [13]. Postprandial lipemia is an emerging cardio-metabolic risk factor, independently of fasting lipid levels[14], and dietary fat lipotoxicity has been implicated

in liver injury in NASH[3,4,5]: Hypothesizing dietary fat lipotoxicity may mediate the impact of TM6SF2 on liver disease and cardio-metabolic risk in NAFLD, we assessed the effect of TM6SF2 C>T variant on postprandial lipoprotein metabolism and on glucose homeostasis in biopsy-proven NAFLD patients and healthy controls.

METHODS

Participants. There are no data on the impact of TM6SF2 C>T variant on postprandial lipoprotein metabolism and glucose homeostasis. Based on available data on the impact of TM6SF2 C>T variant on fasting lipid levels [6,7,8,10] and on the impact of NAFLD on lipoprotein and glucose metabolism [12,15], considering a type I error of 0.05 and a type II error of 0.20: at least 18 T-allele carriers per arm were needed to detect a significant difference in parameters related to lipoprotein metabolism (IAUC triglyceride and LDL-C) and glucose homeostasis (whole-body and tissue insulin sensitivity, β-cell function) within different TM6SF2 genotypes in NAFLD patients.

As obesity, dyslipidemia and diabetes may modify the effect of TM6SF2 C>T variant on glucose/lipid metabolism, on adipokines and on liver disease, subjects with obesity(BMI ≥30 kg/m2), diabetes(fasting plasma glucose ≥126 mg/dl *or* plasma glucose ≥200 mg/dL at +2h on OGTT or antidiabetic drugs), overt dyslipidaemia(fasting serum cholesterol ≥ 200 mg/dL or plasma triglyceride ≥ 200 mg/dL) or clinical signs/symptoms of CVD were excluded.

Sixty nonobese nondiabetic normolipidemic biopsy-proven NAFLD patients referred to two Hepato-Metabolic Clinics were included (criteria for diagnosis of NAFLD are detailed in *Supplementary Online Appendix*). Each pathological feature of liver biopsy was read by a single pathologist (RP) blinded to the patient clinical-biochemical characteristics and scored according to the NASH Clinical Research Network criteria; NASH was defined according to current recommendations[1]**.**

Sixty randomly identified healthy controls, i.e. nondiabetic nonobese normolipidemic individuals without evidence of CVD, randomly selected from a population-based cohort study, matched for TM6SF2 C>T genotype, age, gender, BMI, and waist circumference were included[12]. Criteria to rule out NAFLD in controls are detailed in *Supplementary Online Appendix.*

Patients and controls were characterized for lifestyle habits, routine biochemistry, adipokine profile, markers of inflammation and endothelial dysfunction, as detailed below. Homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as the product of the fasting glucose and insulin concentration divided by 22.5[16].

Participants gave their consent to the study, which was conducted according to the Helsinki Declaration and was approved by the Institutional Review Board of San Giovanni Battista Hospital, Turin, Italy

Genetic analyses.

Genotyping for *TM6SF2* rs58542926 C/T SNP utilized the real-time allele discrimination method, using TaqMan Allelic Discrimination Assay (Applied Biosystems, Foster city, CA). The TaqMan genotyping reaction was run on an 7300HT Fast Real-Time PCR (Applied Biosystem).

We also genotyped our population for the *PNPLA3* SNP rs738409 C/G and for *apoE* genotype, which have been previously linked to both NAFLD and lipid metabolism[17], to assess their interference with outcome variables (detailed in *Supplementary Online Appendix).*

Dietary and physical activity record.

Participants filled in the validated European Prospective Investigation into Cancer and Nutrition (EPIC) 7-day alimentary questionnaire, and the Minnesota-Leisure-Time-

Physical-Activity questionnaire, and data were analyzed as described in *Supplementary Online Appendix.*

Anthropometry.

Percent body fat was estimated by the bioelectrical impedance analysis (BIA) method (TBF-202, Tanita, Tokyo, Japan), closely correlating with dual X ray absorption[18]. Abdominal visceral fat area (cm^2) was estimated using Stanforth equations, validated against computed tomography in black and white Caucasians[19].

Indirect calorimetry and substrate oxidation rates.

After an overnight (12 h) fast, participants underwent indirect calorimetry measurement of oxygen consumption (VO2) and carbon dioxide production (VCO2) using an open circuit indirect calorimeter with a ventilated-hood system (Deltatrac™ II, Datex Instrumentarium Corp., Helsinki, Finland)(see *Supplementary Online Appendix*). Whole-body respiratory quotient (RQ) and non-proteic RQ(npRQ) wERE calculated as VCO2/VO2. Resting energy expenditure (REE), and whole-body CHO oxidation (CHO_{ox}) and fat oxidation rates (Fat_{ox}) were calculated from VO2 and VCO2 by using stoichiometric equations and appropriate energy equivalents[20]. REE and substrate oxidation rates were corrected for fat-free mass(FFM).

Markers of cardiovascular risk/endothelial dysfunction and adipokines.

Serum C-reactive protein (CRP), soluble adhesion molecules E-selectin and intercellular adhesion molecule (ICAM)-1 were measured as validated markers of CVD risk, endothelial dysfunction, and subclinical atherosclerosis[21,22](detailed in *Supplementary Online Appendix).* Circulating adipokines adiponectin, tumor necrosis factor(TNF)-α, resistin and leptin were measured by immunoenzymatic methods (see *Supplementary Online Appendix).*

Oral glucose tolerance test (OGTT)-derived indexes of glucose homeostasis.

Participants underwent a standard 75-g OGTT and indexes of glucose homeostasis were calculated (detailed in *Supplementary Online Appendix*). Whole body oral glucose insulin sensitivity index (OGIS), and hepatic and muscle insulin resistance indexes were calculated as previously proposed and validated against clamp in nondiabetic subjects[23, 24].

Adipose tissue insulin resistance (adipo-IR) index was calculated as fasting non-esterified fatty acids (NEFA) x fasting insulin[15].

The Minimal Model technique was used to calculate the following indexes of β-cell function: the insulinogenic index (IGI) and the CP-genic index (CGI) and the 2 integrated indexes of β-cell function disposition index (DI) and adaptation index (AI), which relate β-cell insulin secretion to insulin resistance. DI and AI were previously validated against frequently-sampled intravenous glucose tolerance test (FIVGTT) in NAFLD and in nondiabetic subjects[21,25] and reliably predict T2DM development[26].

Incretin effect. To assess if differences in β-cell function were related to a reduced incretin stimulatory effect on β-cell, a FIVGTT was performed and the incretin effect, i.e., the effectiveness of ingested glucose in stimulating β-cell insulin secretion compared to intravenous. glucose, was assessed (see *Supplementary online Appendix).*

Oral fat tolerance test (OFTT).

Participants underwent a 10-hour oral fat tolerance test(OFTT)[14] with measurement of the following parameters (methods detailed in *Supplementary Online Appendix*):

1)plasma total cholesterol (Chol), triglyceride (Tg), NEFA and HDL-C

2)triglyceride-rich lipoproteins (TRLP) subfractions and LDL: TRLP were isolated through preparative ultracentrifugation and their total Tg and Chol content were subsequently measured as described in *Supplementary Online Appendix*.

Two VLDL subfractions with decreasing Sf values (VLDL1: Sf>100; VLDL2: Sf =20-100) were separated and their Chol and Tg content was determined (see *Supplementary Online Appendix).*

VLDL apoB48 and apoB100 were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using 3,9% gel (detailed in *Supplementary Online Appendix*).

LDL cholesterol content was measured with a standardised homogeneous enzymatic colorimetric method in order to avoid triglycerides effects on LDL determination (Sentinel) (see *Supplementary Online Appendix).*

3)lipid-induced oxidative stress: oxidized low-density lipoproteins (oxLDLs). LDL conjugated dienes, validated markers of oxLDLs, were determined by capillary electrophoresis(detailed in *Supplementary Online Appendix).*

4) Glucose-dependent insulinotropic polypeptide (GIP), adiponectin and resistin.

GIP is an emerging modulator of lipid metabolism independently of its incretin effect on pancreatic β-cell function. Dietary fat is the most potent stimulator of GIP secretion [27], and TM6SF2 protein is expressed by human intestinal cells[12]; furthermore, acute and chronic administration of GIP, but not of glucagon-like peptide(GLP)-1, reduces fat oxidation and energy expenditure[28], induces adipocyte dysfunction and proinflammatory adipokine secretion[29], and promotes development of obesity-associated metabolic disorders[30], including NAFLD, which were all reversed by GIP antagonists[28].

Plasma GIP, as well as resistin and adiponectin, which have been linked to both liver disease severity and lipoprotein metabolism in NAFLD, were measured as detailed in *Supplementary Online Appendix*.

Statistical analysis

Differences across groups were analyzed by ANOVA followed by Bonferroni correction, when variables were normally distributed; otherwise the Kruskal-Wallis test, followed by the post hoc Dunn test, was used. Normality was evaluated by Shapiro-Wilk test. Fisher or chi-square test were used to compare categorical variables, as appropriate. Hardy–Weinberg equilibrium was assessed using $\chi 2$ test.

To adjust for multiple comparison testing, the Benjamini-Hochberg False Discovery Rate correction was applied to raw p-values in all comparisons; significance was set at an adjusted p-value threshold of 0.05[31].

The area under the curve(AUC) and incremental AUC(IAUC) of parameters measured during the OFTT and the OGTT were computed by the trapezoid method.

Due to the low prevalence of TM6SF2 TT homozygotes and to the overlapping clinical characteristics with heterozygous CT carriers, TM6SF2 TT carriers were combined with CT heterozygotes for group comparisons.

Differences were considered statistically significant at $p<0.05$.

Analysis of dietary, anthropometric and metabolic parameters and of genetic polymorphisms was made using Spearman correlation test to assess correlation among different variables.

Based on available evidence [6,7,8,10], TM6SF2 C>T variant was modelled as a dominant model of inheritance, that is, quantitative predictor variables reflecting the number of risk alleles (0, 1, or 2).

When a relation was found on univariate analysis, multivariate logistic regression was used to identify independent predictors of selected outcome variables of interest, namely:

-for liver histology: the presence of NASH and of advanced (stage 3) fibrosis

- for CVD risk: serum CRP and endothelial adhesion molecules E-selectin and ICAM-1;

-for whole-body nutrient oxidation rates: CHO_{ox} and fat_{ox}.

-for glucose homeostasis: OGTT-derived parameters of whole-body/tissue insulin resistance and of β-cell function;

-for postprandial lipid metabolism: the IAUC of triglyceride, LDL-C, oxLDL and of of main triglyceride-rich lipoprotein subfractions.

For this analysis, continuous variables were divided into quartiles and independent predictors of the highest quartile of outcome variable were asesssed, after after log transformation of skewed data. The independent predictors were those variables found to be related to the outcome variables on univariate analysis.

Data were expressed as mean ± SEM, unless otherwise specified. (STATISTICA software, 5.1, Statsoft Italia, Padua, Italy)

RESULTS.

Subjects characteristics

Main features of patients and controls grouped according to TM6SF2 C>T genotype are reported in **Table 1**.

In study participants, the prevalence TM6SF2 CC homozygotes was 64%, of CT heterogygotes was 34%, of TT carriers was 2%;

The distribution of TM6SF2 CT genotype was in Hardy-Weinberg equilibrium(6, 7, 8).

NAFLD as a group had higher HOMA, serum CRP and endothelial adhesion molecules E-selectin and ICAM-1 and lower HDL-C and adiponectin than controls. Within NAFLD patients and controls, TM6SF2 CT/TT carriers showed lower serum CRP and endothelial adhesion molecules than TM6SF2 CC genotype carriers (**Table 1**).

Among NAFLD patients, 42% had NASH and 16% had advanced fibrosis. TM6SF2 T-allele carriers had more severe liver histology than their counterpart genotype (**Table 1**).

Alimentary record.

There was no difference in daily total energy, macro- and micro-nutrient, types of fat and

antioxidant vitamin intake between patients with NAFLD and controls and among different TM6SF2 genotypes (not shown).

Indirect calorimetry.

While TM6SF2 C>T variant did not affect REE, the proportion of energy derived from fat and CHO oxidation differed between TM6SF2 genotypes: TM6SF2 T-allele carriers had lower RQ and npRQ, indicating they oxidize more fat and less CHO that CC homozygotes(**Table 1***).*

OGTT-derived indexes of glucose homeostasis

The time course of plasma glucose and serum insulin during the OGTT is reported in **Figure S1**. In patients and controls, TM6SF2 T-allele carriers showed higher hepatic and adipose insulin resistance but enhanced muscle insulin sensitivity than CC homozygotes TM6SF2 CT/TT genotype displayed also impaired pancreatic β-cell function and incretin effect than CC homozygotes(**Table 2**).

Oral fat tolerance test.

Within patients and controls, TM6SF2 CT/TT genotype showed lower postprandial Tg, VLDL1-Tg, NEFA and oxLDL responses, a higher increase in postprandial cholesterol content in VLDL1 and VLDL2 subfractions of intestinal and hepatic origin, and a slight but statistically significant postprandial LDL-C decrease as compared with TM6SF2 CC genotype (**Table 3, Figure 1 panel A-D, Supplementary Figure S2**).

TM6SF2 CT/TT genotype showed also lower postprandial GIP and higher resistin responses than homozygous CC carriers (**Table 3, Figure 1 panel F-G**).

Independent predictors of outcome variables on multiple logistic regression analysis

Liver histology: NASH was independently predicted by by IAUC VLDL1-Ch (OR=1.60, 95% CI: 1.1-2.2, p=0.009), while advanced (stage 3) fibrosis was predicted by IAUC adiponectin (OR=1.41, 95%CI: 1.1-2.0, p=0.021) and IAUC VLDL1-Ch(OR=1.53, 95%CI: 1.1-2.2, p=0.010).

Circulating markers of CVD risk: IAUC triglyceride and IAUC oxLDLs independently predicted **C-reactive protein** (OR=1.51, 95%CI: 1.05-2.65, p=0.006 and β=1.48, 95%CI: 1.08-2.54, p=0.005, respectively), **E-selectin** (OR=1.56, 95%CI: 1.11-2.61, p=0.002 and OR=1.54, 95%CI: 1.19-2.63, p=0.0009, respectively), and **ICAM-1**(OR=1.54, 95%CI: 1.18-2.78, p=0.009 and OR=1.52, 95%CI: 1.07-2.77, p=0.010, respectively).

Whole-body Fat_{ox} was independently predicted by IAUC adiponectin (OR=1.49, 95%CI: 1.14-2.59, p=0.002). and IAUC GIP (β=0.49, 95%CI: 0.18-0.88, p=0.012).

The independent determinants of **OGTT-related glucose homeostasis** parameters and of **posptrandial lipoprotein and adipokine responses** during oral tat tolerance test are reported in **Table 4**.

DISCUSSION

The main findings of our study are the following:

1)TM6SF2 C>T variant modulates postprandial lipid metabolism: despite similar fasting lipid levels, TM6SF2 CT/TT carriers show lower postprandial triglyceride, NEFA and oxLDL responses, higher HDL-C levels, and a cholesterol redistribution from LDL to larger intestinal and hepatic TRLPs subfractions. TM6SF2 T-allele carriers have also higher incretin GIP and resistin elevations after fat ingestion.

2)Postprandial plasma VLDL1-Ch elevation independently predicts the severity of liver histology in NAFLD, while triglyceride and oxDLD responses were independently associated with markers of CVD risk.

3)TM6SF2 C>T variant affects tissue insulin resistance, pancreatic ß-cell function, and whole-body substrate oxidation rate, the latter possibly through modulation of GIP response to dietary fat.

Postprandial lipemia is an independent cardio-metabolic risk factor in the Western world and, consistently, individuals spend most of the day in the postprandial phase rather than in fasting conditions[14]. The effect of TM6SF2 variant on dietary fat metabolism may contribute to the dual and opposite effect of this SNP on liver disease severity and on CVD risk in NAFLD[32]: following fat ingestion, TM6SF2 T-allele carriers showed a shift in cholesterol content from LDL to larger intestinal and hepatic VLDL subfractions, which are preferentially taken-up by liver cells and adipocytes through the low-density lipoprotein receptor-related protein (LRP)[33, 34] and the VLDL-receptor(VLDLR)[35], thereby triggering hepatocyte apoptosis and adipocyte dysfunction[33,34,35]. The independent association of postprandial VLDL-Ch response with liver histology is consistent with recent data, demonstrating an important role for TRLP uptake in promoting high fat-induced liver injury[36] and linking cholesterol concentration in VLDL subclasses to hepatic cholesterol content, inflammation, and fibrosis[37].

These findings suggest TM6SF2 T-allele-associated postprandial lipoprotein pattern may divert toxic cholesterol away from the vessel walls into the liver and adipose tissue, enhancing liver injury and adipose dysfunction and protecting from CVD.

The independent association of CVD risk markers with postprandial triglyceride and oxLDL responses, which were lower in TM6SF2 T-allele carriers, is also consistent with an important role for postprandial lipoprotein metabolism in mediating the

cardioprotective role of T-allele observed in large epidemiological studies[7,10]

The lower postprandial triglyceride response in TM6SF2 T-allele carriers may be due to lower fat absorption, or greater chylomicron clearance. The lower increase in NEFA is not consistent with greater chylomicron clearance, which would have increased plasma NEFA through spillover. Additionally a recent report showed TM6SF2 T-allele impairs triglyceride processing and secretion in enterocytes[38], confirming a reduced triglyceride absorption may underlie the lower postprandial lipemia observed in TM6SF2 T-allele carriers.

If confirmed by larger studies, these findings may have therapeutic implications, as cholesterol-lowering interventions may reduce cholesterol hepatotoxicity in TM6SF2 T-allele carriers, irrespective of fasting cholesterol levels.

We also evaluated the impact of TM6SF2 SNP on glucose homeostasis, as both NAFLD and TM6SF2 C>T variant have been associated with an increased risk of type 2 diabetes[2,11] . TM6SF2 gene variant affected tissue insulin sensitivity and pancreatic β-cell function: TM6SF2 T-allele was associated with an impaired incretin effect and ß-cell function, possibly via a reduced incretin secretion or action on ß-cells, which express TM6SF2 protein[13]. These findings may help select NAFLD carriers of TM6SF2 at-risk genotype, who are also at higher risk of T2DM for targeted preventive interventions improving β-cell dysfunction, including incretin mimetics.

An intriguing finding was the impact of TM6SF2 SNP on muscle insulin sensitivity and whole-body fat oxidation rates, both effects related to postprandial adiponectin and GIP responses to fat (**Table 4**).

Consistent with our data, adiponectin stimulates muscle fat oxidation and insulin sensitivity, while GIP potently reduces energy expenditure and fat oxidation[39]. The link between TM6SF2 and incretins and the role of GIP antagonism to enahnce fat oxidation and insulin sensitivity warrant future investigation. In the meantime, it should be noted

that GIP increase induced by dipeptidyl peptidase-IV (DDP-IV) inhibitors, currently evaluated in NAFLD, may attenuate the benefits of glucagon-like peptide(GLP)1 elevation[40].

In conclusion, a maladaptive response to a chronic, daily, repetitive metabolic challenge like fat ingestion may link TM6SF2 C>T variant to liver injury and cardio-metabolic disease in NAFLD. Future research should unravel underlying molecular pathways in different tissues and organs, allowing therapeutic interventions tailored to individual risk profile and mechanism of injury[41,42,43].

Strengths of our study are the careful selection and thorough characterization of participants. Limitations are the small number of subjects and the cross-sectional design, which prevents any causal inference between TM6SF2 variant and the abnormalities in lipid and glucose metabolism and requires confirmation by larger follow-up studies.

A further caveat is that we did not measure directly hepatic and muscle insulin sensitivity but rather estimated them from the time course of glucose and insulin during the OGTT. This method assumes a similar intestinal glucose absorption rate across TM6SF2 genotypes, as a faster glucose absorption rate in TM6SF2 T-allele carriers would cause a steeper increase and an earlier peak and fall in plasma glucose regardless of any actual differences in tissue insulin sensitivity: however, the visual inspection of plasma glucose curve during the OGTT (**Supplementary Figure S1**) shows a similar slope in the 0'-30' ascending limb of the curve across TM6SF2 genotypes and the same peak time (+60'), making differences in glucose absorption very unlikely to occur.

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Author's contributions:

Giovanni Musso: designed research, conducted research, analyzed data, wrote paper, has primary responsibility for final content;

Ugo Cipolla: conducted research, analyzed and discussed data, approved final version of the paper;

Maurizio Cassader: conducted research, analyzed and discussed data, approved final version of the paper;

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Table 1. Main clinical, biochemical and histological parameters of biopsy-proven NAFLD patients and controls grouped according to TM6SF2 C/T polymorphism (n=120).

	Controls			**NAFLD**		
	TCM6F2 CC (n=40)	**TCM6F2 CT/TT (n=20)**	**P**	**TM6SF2 CC (n=40)**	**TM6SF2 CT/TT (n=20)**	**P**
Age (years)	42±2	42±2	0.851	42±2	40±2	0.851
Sex (%males)	68	65	0.693	68	65	0.693
BMI (kg/m^2)	25.6±0.5	25.9±0.6	0.731	25.6±0.5	25.8±0.6	0.690
Fat mass(%)	22±2	22±2	0.872	23±2	22±2	0.232
Waist (cm)	89±3	90±4	0.482	89±2	90±2	0.426
WHR	0.91±0.02	0.91±0.03	0.756	0.92±0.03	0.92±0.03	0.731
AVF(cm2)	99±5	103±6	0.731	101±5	97±6	0.832
Smokers (%)	31	30	0.410	33	31	0.390
Systolic BP (mmHg)	118±3	123±2	0.291	121±2	127±2	0.280
Diastolic BP (mmHg)	80±2	84±2	0.130	83±2	87±5	0.122
AST (U/L)	15±1	16±2	0.591	32±2	41±4‡	0.131
ALT (U/L)	19±2	2312	0.678	70±5	88±6‡	0.111
GGT (U/L)	35±5	43±4	0.702	89±16	108±18	0.089
Tg (mg/dL)	98±211	86±10	0.879	94±17	85±13	0.561
Total C (mg/dL)	179±9	168±7	0.311	187±11	173±12	0.132
HDL-C (mg/dL)	54±2	55±2	0.210	52±2§	54±2§	0.118
LDL-C (mg/dL)	103±6	94±6	0.131	107±5	95±10	0.210
Glucose(mg/dL)	99±3	90±3	0.394	100±10	90±7	0.273
Insulin (μU/mL)	7.2 ±1.8	6.3±1.2	0.569	13.7±3.8	15.9±6.4	0.543
HOMA-IR	1.9±0.9	1.3±0.8	0.298	3.55±1.1	2.9±0.90	0.220
METS(h/week)	21.2±1.0	22.2±1.7	0.413	22.7±1.5	21.9±1.4	0.639
RQ	0.81±0.01	0.77±0.01	**0.001**	0.81±0.01	0.78±0.01	**0.003**

npRQ	0.81±0.02	0.76±0.01	**0.001**	0.82±0.01	0.77±0.01	**0.0009**
REE(kcal/24h/kg/FFM)	29.5±1.8	29.9±2.0	0.711	29.7±1.5	28.4±1.7	0.302
Fat_{ox} (mg/kg/FFM/min)	1.23±0.05	1.54±0.05	**0.0009**	1.22±0.06	1.50±0.08	**0.002**
CHO_{ox} (mg/kg/FFM/min)	2.00±0.10	1.42±0.11	**0.001**	1.99±0.11	1.45±0.10	**0.002**
Hs-CRP(mg/L)	1.9±0.2	1.1±0.4	**0.009**	3.1±02†	2.0±02§	**0.001**
E-selectin (ng/mL)	31.1±3.1	20.1±4.6	**0.010**	51.3±4.8†	28.9±3.1§	**0.002**
ICAM-1(ng/mL)	239.1±4.6	191.8±5.3	**0.028**	285.1±5.2†	228.6±6.0§	**0.009**
TNF-α (pg/mL)	1.20±0.18	1.08±0.21	0.512	1.18±0.17	0.99±0.25	0.471
Leptin (pg/mL)	1830±399	1793±224	0.430	1746±275	1914±201	0.711
ApoE Genotype(%)						
2-3	16	14	0.573	14	16	0.689
3-3	66	67	0.312	67	67	0.911
3-4	18	19	0.690	19	17	0.892
PNPLA3 (%)						
CC	41	55	0.671	41	55	0.671
CG	41	33	0.312	41	33	0.312
GG	8	12	0.218	8	12	0.218
abdominal obesity (%)	17	20	0.691	17	20	0.691
IGR(%)	19	8	0.231	21	10	0.289
Hypertension(%)	30	27	0.379	51	49	0.592
Low HDL-C(%)	13	9	0.398	16	9	0.401
High Tg(%)	13	9	0.412	14	8	0.379
Met sy (%)	37	29	0.311	40§	31§	0.297
Steatosis(% hep.)	-	-	-	25±3	32±4	0.168
NAFLD activity score			-	2.0±0.2	4.0±0.3	**0.0001**
Fibrosis stage	-	-	-	0.2±0.1	1.0±0.2	**0.0001**
NASH(%)	-	-	-	31	61	**0.045**

* $p<0.05$ vs. controls

† $p<0.01$ vs. controls

§ $p<0.05$ vs. controls bearing the same genotype

‡ $p<0.01$ vs. controls bearing the same genotype

¶ $p<0.05$ vs. controls bearing the counterpart genotype

# $p<0.01$ vs. controls bearing the counterpart genotype

Abbreviations. AVF: abdominal visceral fat area; BP: blood pressure; total C: total cholesterol; $Fat_{ox:}$ fat oxidation rates. FFM: tat-free mass; hs-CRP: highly sensitive C-reactive protein; CHO: carbohydrates; RQ: respiratory quotient; npRQ: nonproteic respiratory quotient; REE: resting energy expenditure; WHR: waist-on-hip ratio; Tg: triglyceride; IGR: impaired glucose regulation; HOMA-IR: homeostasis model assessment of insulin resistance; ICAM: intercellular adhesion molecule; METS: Metabolic equivalent of activity; Met sy: metabolic syndrome according to the joint statement of AHA, IDF and NHLBI;

MTP: microsomal triglyceride transfer protein; SREBF: sterol regulatory element-binding factor;

Met Sy: metabolic syndrome according to the joint statement of AHA, IDF and NHLBI, requires the presence of ≥3 of the following criteria:

-abdominal obesity: waist circumference≥102 cm(males) and ≥88 cm(females)

-high triglycerides: ≥150 mg/dL (1.7 mmol/L) or on drug treatment for elevated triglycerides

-low HDL-C: <40 mg/dL (1.0 mmol/L) (males) or <50 mg/dL (1.3 mmol/L) (females) or on drug treatment for reduced HDL-C

-hypertension: systolic BP≥130 and/or diastolic BP≥85 mm Hg or on drug treatment

-high fasting plasma glucose (FPG): FPG ≥100 mg/dL (5.6 mmol/L) or on drug treatment for elevated glucose.

Table 2. OGTT-derived Indexes of Glucose Homeostasis in patients with biopsy-proven NAFLD and controls, grouped according to TM6SF2 rs58542926 C/T genotype (n=120).

	TM6SF2 C/T genotype							
		Controls			NAFLD			
		CC (n=40)	CT/TT (n=20)	p	CC (n=40)	CT/TT (n=20)	p
OGIS ($ml \cdot min^{-1} \cdot m^{-2}$)		427.9± 13.5	442.6±15.1	0.318	385.5± 7.4*	392.2± 11.0*	0.810
Hepatic IR ($g/dL_{glucose} \cdot \mu U/mL_{Ins} \cdot min^{-2}$)		2615.7± 126.4	3298.4±173.5	**0.001**	4180.1± 107.4*	4779.7± 182.1†	**0.002**
Muscle IS		0.014± 0.002	0.021± 0.001	**0.028**	0.012± 0.001	0.018± 0.002	**0.002**
Adipose IR (mmol/L/pmol/L)		21.2±2.0	30.1±1.4	**0.0004**	48.6±4.2†	88.4±6.8†	**0.0001**
Hepatic extraction (%)		74±3	72±4	0.414	73±5	69±7	0.582
IGI ($\mu U_{insulin} \cdot g^{-1}_{glucose}$)		187±11	112±14	**0.009**	171±19§	106±13†	**0.001**
CGI ($ng_{C\text{-}pep} \cdot g^{-1}_{glucose}$)		511±12	401±11	**0.0009**	502±13§	394±16†	**0.001**
DI ($\mu U_{insulin} \cdot g^{-1}_{glucose} \cdot ml^{-1} \cdot m^{-2}$)		80124± 4318	48615± 4379	**0.001**	52136± 3615§	37639± 1713†	**0.0001**
AI ($ng_{C\text{-}pep} \cdot g^{-1}_{glucose} \cdot ml^{-1} \cdot m^{-2}$)		220709±12138	175241± 8136	**0.009**	189420± 8372§	142671± 9139†	**0.001**
Incretin effect (%)		72.6±3.2	47.3±2.9	**0.0002**	70.9± 3.1	45.2± 4.1	**0.0001**

* p<0.05 vs. controls

† p<0.01 vs. controls

§ p<0.05 vs. controls bearing the same genotype

‡ p<0.01 vs. controls bearing the same genotype

¶ p<0.05 vs. controls bearing the counterpart genotype

# p<0.01 vs. controls bearing the counterpart genotype

Data are presented as mean ± SEM, unless otherwise specified.

Abbreviations: OGIS: oral glucse insulin sensitivity index; IR: insulin resistance;

IS: insulin sensitivity; IGI: insulinogenic index; CGI: Cp-genic index;

DI: Disposition Index; AI: Adaptation Index

Table 3. Oral fat tolerance test parameters in patients with NAFLD and controls grouped according to TCM6F2 rs58542926 C/T genotype (n=120).

	Controls			**NAFLD**		
Parameter	**TCM6F2 CC (n=40)**	**TCM6F2 CT/TT (n=20)**	**P**	**TCM6F2 CC (n=40)**	**TCM6F2 CT/TT (n=20)**	**P**
Fasting Tg(mg/dL)	98±11	86±10	0.812	94±15	85±18	0.513
IAUC Tg (mg/dL x hr)	141±12	79±10	**0.001**	525±21†	297±20†	**0.00001**
Fasting NEFA (mmol/L)	0.35±0.23	0.47±0.28	0.712	0.50±0.29	0.63±0.31	0.711
IAUC NEFA (mmol/L x hr)	1.93±0.27	0.82±0.15	**0.00009**	5.24±0.22†	2.31±0.28§	**0.0001**
Fasting VLDL1-Tg (mg/dL)	42±9	40±10	0.812	52±12	36±10	0.201
IAUC VLDL1-Tg (mg/dL x hr)	408±29	123±14	**0.0001**	922±37†	497±31§	**0.00002**
Fasting VLDL2-Tg (mg/dL)	30±7	31±7	0.813	36±8	42±9	0.312
IAUC VLDL2-Tg (mg/dL x hr)	56±10	89±13	0.301	137±14	131±19	0.611
Fasting VLDL1-Ch (mg/dL)	10±2	12±2	0.812	14±4	16±4	0.713
IAUC VLDL1-Ch (mg/dL x hr)	41±4	92±7	**0.00009**	97±9§	199±11†	**0.000001**
Fasting VLDL2-Ch (mg/dL)	15±3	13±3	0.712	18±3	20±4	0.611
IAUC VLDL2-Ch (mg/dL x hr)	11±1	32±2	**0.00009**	37±2§	108±4§	**0.000001**
Fasting LDL-C(mg/dL)	103±6	94±6	0.131	107±5	95±10	0.210
IAUC LDL-C (mg/dL x hr)	-10±2	-24±2	**0.003**	-20±3 # §	-51±3†	**0.0001**
Fasting VLDL1 ApoB48 (mg/dL)	2.1±0.4	2..0±0.5	0.812	2.7±0.9	2.4±0.9	0.511
IAUC VLDL1 ApoB48 (mg/dL x hr)	4.5±0.9	1.9±0.5	**0.0002**	8.7±1.4†	4.3±1.0§	**0.00001**
Fasting VLDL2 ApoB48 (mg/dL)	1.8±0.4	1.5±0.4	0.509	2.3±0.6	2.1±0.7	0.421
IAUC VLDL2 ApoB48 (mg/dL x hr)	1.5±0.3	2.9±0.5	**0.008**	1.6±0.3	5.8±0.6†	**0.0001**

Fasting VLDL1ApoB100(mg/dL)	3.7±1.0	3.5±1.1	0.712	4.5±1.6	4.2±1.7	0.913
IAUC VLDL1 ApoB100 (mg/dL xhr)	10.0±1.5	3.9±0.9	**0.00009**	22.4±3.5†	11.7±2.9§	**0.00001**
Fasting VLDL2 ApoB100 (mg/dL)	3.7±0.7	3.2±0.9	0.802	5.2±0.9	4.8±1.1	0.611
IAUC VLDL2 ApoB100 (mg/dL x hr)	4.6±0.9	8.3±1.0	**0.015**	13.8±1.9†	24.5±2.6†	**0.00001**
Fasting LDL C.D. (uA 234 nm/uA 200 nm x 100)	7.3±1.6	7.9±1.8	0.902	7.5±1.8	7.1±1.6	0.616
IAUC LDL C.D. (uA 234 nm/uA 200 nm x 100 x hr)	2.1±0.1	0.8±0.2	**0.0009**	15.1±1.0†	5.2±1.2*	**0.00001**
Fasting HDL-C(mg/dL)	54±2	55±2	0.210	52±2	54±2	0,212
IAUC HDL-C (mg/dL x hr)	-14±2	2±1	**0.0001**	-56±4†	-18±2§	**0.00009**
Fasting GIP (pg/mL)	18.8±6.4	16.5±6.1	0.712	22.1±9.5	11.9±5.2	0.211
IAUC GIP (pg/mL x hr)	571.9±18.5	266.4±20.1	**0.000008**	703.9±20.1†	379.6±24.4	**0.000002**
Fasting adiponectin (ng/mL)	8631±782	9515±812	0.412	6161±572	5575±650	0.713
IAUC adiponectin (ng/mL x hr)	11071±912	12916±926	0.513	1768±246	1536±494	0.423
Fasting resistin(ng/mL)	3.4±0.9	3.1±1.0	0.912	3.8±0.9	3.3±0.9	0.301
IAUC resistin (ng/mL x hr)	0.1±0.1	1.5±0.3	**0.008**	2.8±1.1*	6.4±11.9†	**0.0000001**

Oral fat load parameters of patients with NAFLD and controls according to TM6SF2 genotype. Data are presented as mean ± SEM. Statistically significant P values are written in bold characters.

Abbreviations: IAUC: incremental area under the curve; FFA: free fatty acids; Tg: triglyceride; C.D. : conjugated dienes; Ch: cholesterol;

* $p<0.05$ vs. controls

† $p<0.01$ vs. controls

§ $p<0.05$ vs. controls bearing the same genotype

‡ $p<0.01$ vs. controls bearing the same genotype

¶ $p<0.05$ vs. controls bearing the counterpart genotype

# $p<0.01$ vs. controls bearing the counterpart genotype

Table 4. Independent predictors of parameters related to glucose and lipid metabolism in biopsy-proven NAFLD subjects and matched controls on multivariate logistic regression analysis (n=120).

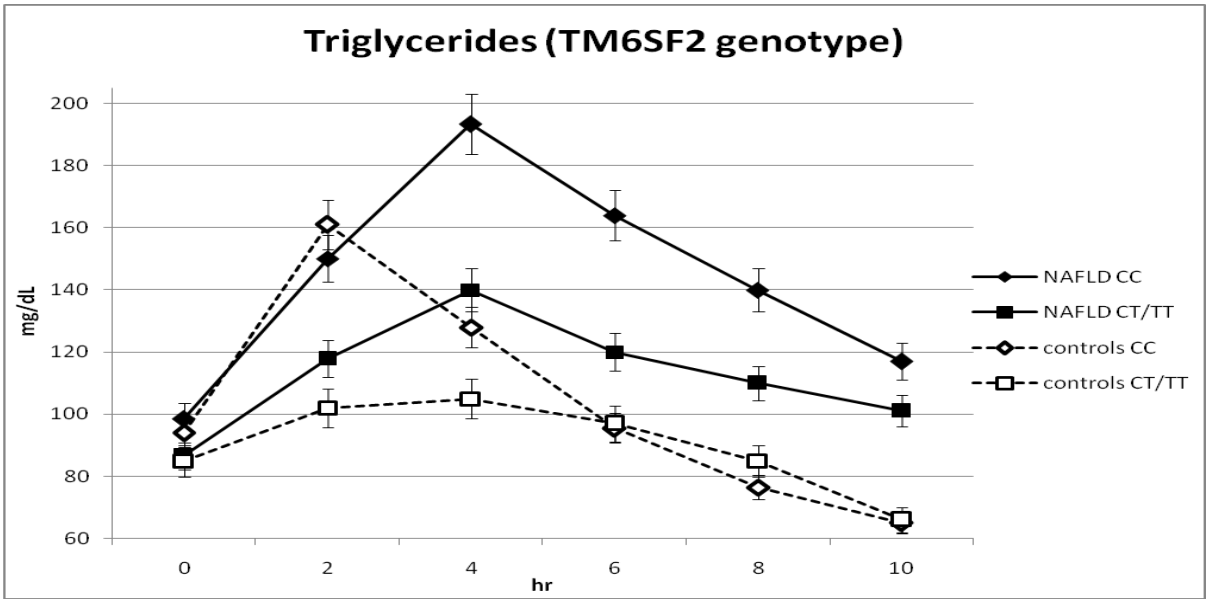
OGTT-related parameters of glucose homeostasis			
Outcome variable	**Independent predictor**	**OR (95% CI)**	**P**
OGIS	IAUC adiponectin	1.50(1.15-2.51)	0.001
Hepatic IR	IAUC adiponectin	0.54(0.16-0.86)	0.001
	IAUC resistin	1.58(1.12-2.63)	0.006
Adipose tissue IR	PNPLA3	1.52(1.06-2.76)	0.008
	IAUC VLDL1-Ch	1.45(1.05-2.59)	0.002
Muscle IS	IAUC adiponectin	1.47(1.07-2.46)	0.011
	IAUC GIP	0.49(0.18-0..91)	0.012
Insulinogenic Index (IGI)	TM6SF2	0.49(0.04-0.83)	0.009
	IAUC adiponectin	1.49(1.04-2.50)	0.004
Disposition Index (DI)	TM6SF2	0.51 (0.16-0.86)	0.001
	IAUC adiponectin	1.49(1.12-2.55)	0.009
CP-genic Index (CGI)	TM6SF2	0.46 (0.11-0.81)	0.001
	IAUC adiponectin	1.68 (1.04-2.50)	0.003
Adaptation Index (AI)	TM6SF2	0.43(0.10-0.70)	0.001
	IAUC adiponectin	1.79(1.23-2.84)	0.002
Incretin effect	TM6SF2	0.45(0.11-0.80)	0.009
	IAUC GIP	0.51 (0.16-0.86)	0.007
Oral fat tolerance test parameters			
Outcome variable	**Independent predictor**	**OR (95% CI)**	**P**
IAUC triglycerides	IAUC adiponectin	0.50(0.14-0.87)	0.003
	TM6SF2	0.47(0.02-0.82)	0.001

IAUC VLDL1-Tg	IAUC adiponectin TM6SF2	0.49(0.13-0.84) 0.43(0.08-0.78)	0.001 0.0009
IAUC VLDL1-Ch	TM6SF2	1.69(1.11-2.81)	0.00002
IAUC VLDL2-Ch	TM6SF2	1.55(1.15-2.60)	0.0009
IAUC VLDL1-apoB100	TM6SF2	0.49(0.13 -0.83)	0.002
IAUC VLDL2-apoB100	TM6SF2	0.45(0.10-0.81)	0.004
IAUC VLDL1-apoB48	TM6SF2	0.44(0.02 -0.80)	0.0001
IAUC VLDL2-apoB48	TM6SF2	0.51(0.06-0.91)	0.023
IAUC LDL-C	TM6SF2 Fasting LDL-C	0.50(0.15-0.85) 1.91(0.36-3.11)	0.003 0.0008
IAUC LDL conjugated dienes	IAUC VLDL1-Tg	1.89(1.23-2.95)	0.0001
IAUC HDL-C	IAUC VLDL1-Tg	0.52(0.17-0.87)	0.009
IAUC GIP	TM6SF2	1.88 (1.21-3.01)	0.001
IAUC resistin	TM6SF2	1.58 (1.13-2.92)	0.012

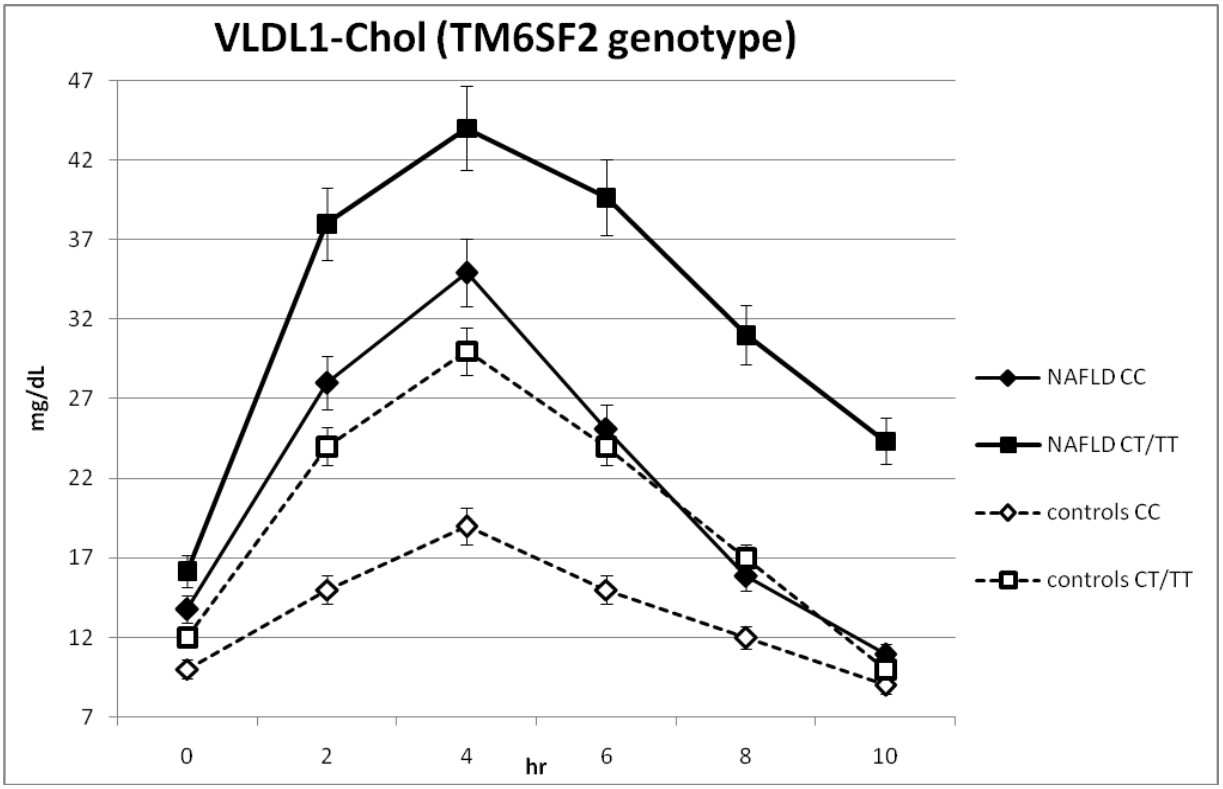
Abbreviations: OGIS: oral glucose insulin sensitivity index; IR: insulin resistance; IS; insulin sensitivity; VLDL: very low density lipoprotein; Ch: cholesterol

Figure 1. Oral fat load test: postprandial responses in plasma triglycerides (panel A), VLDL1 cholesterol (VLDL1-Chol, panel B), VDLD2 cholesterol (VLDL2-Chol, panel C), LDL-C (panel D), oxLDL (panel E), resistin (panel F), and glucose-dependent insulinotropic polypeptide (GIP)(panel G) . Patients and controls were grouped according to TM6SF2 genotype. Data are presented as mean±SEM (N=120).

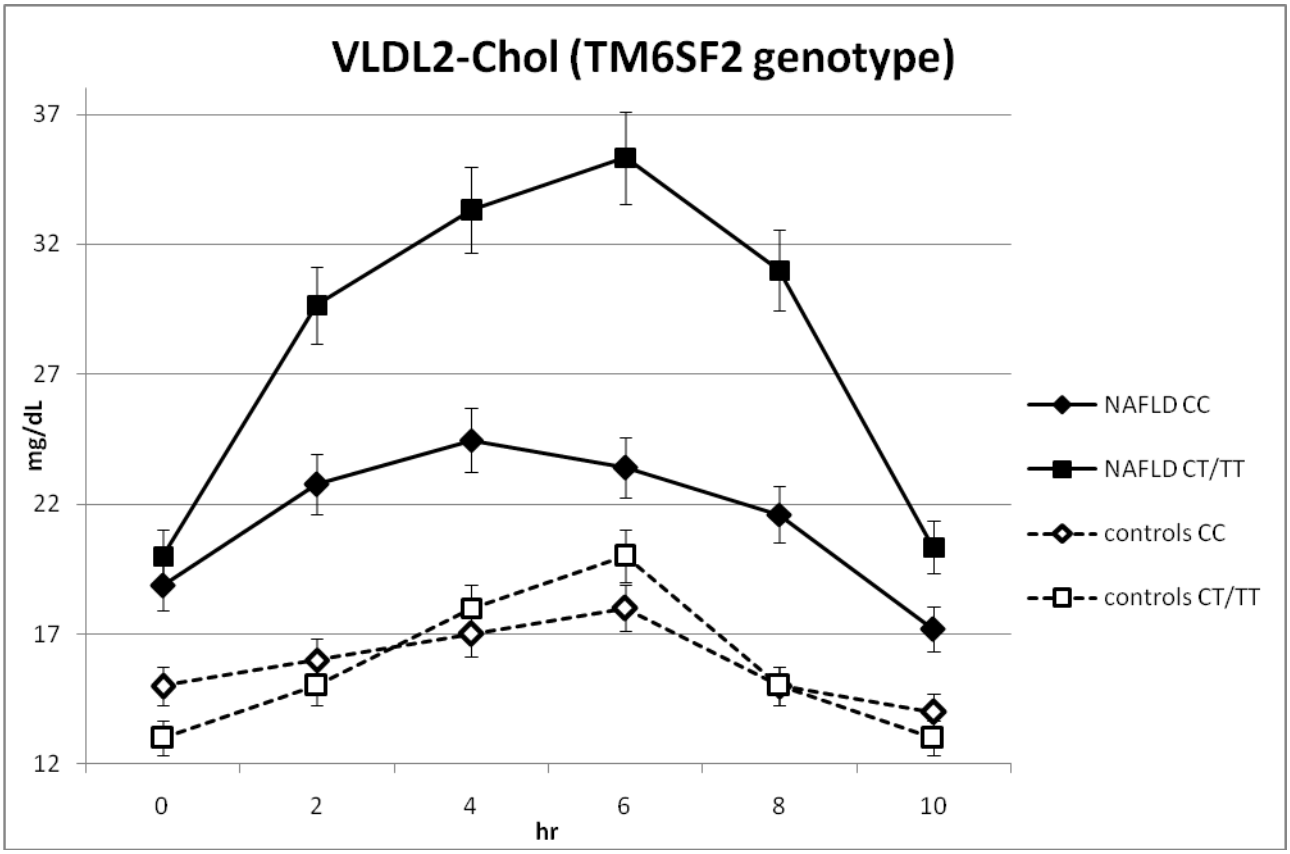
Panel A



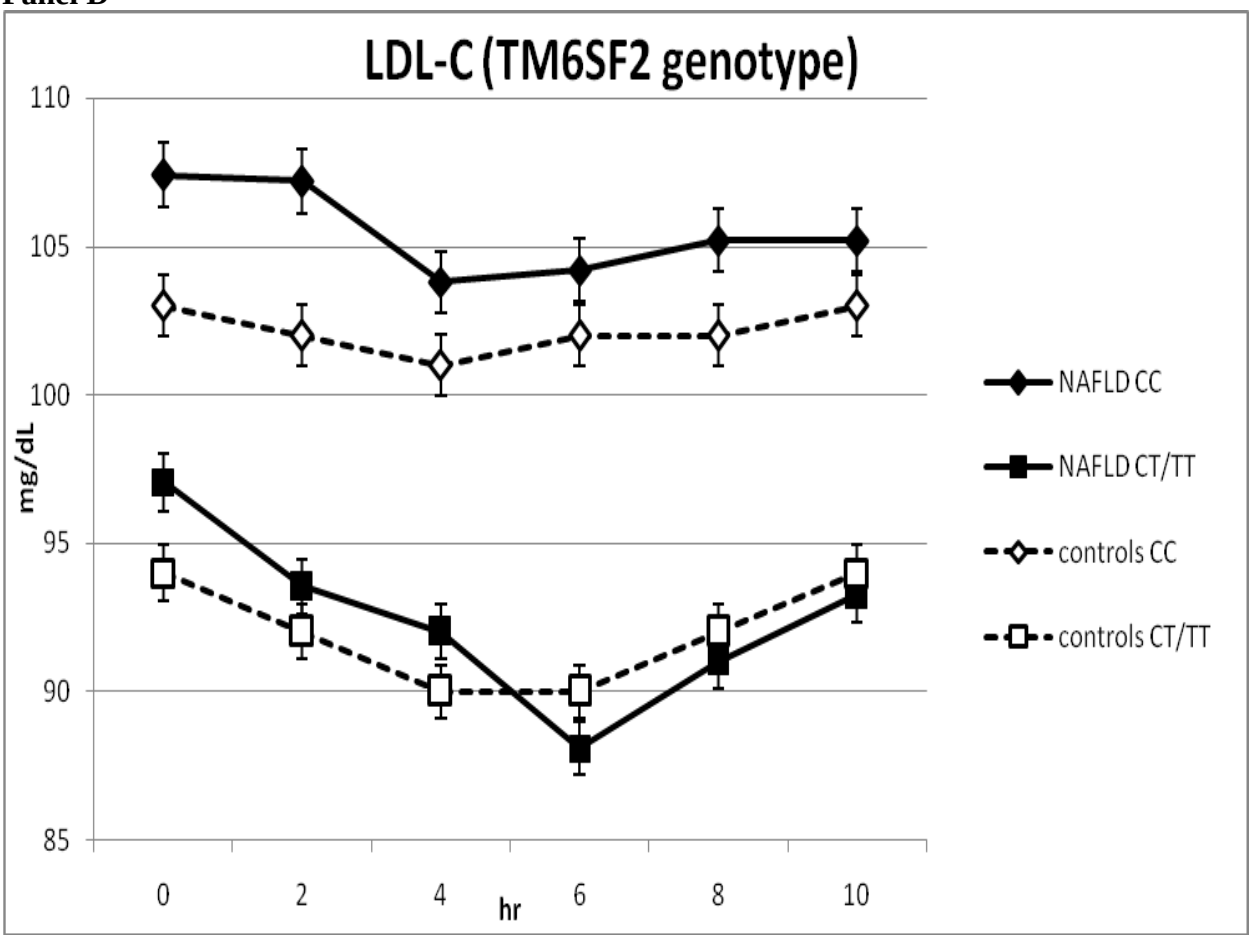
Panel B



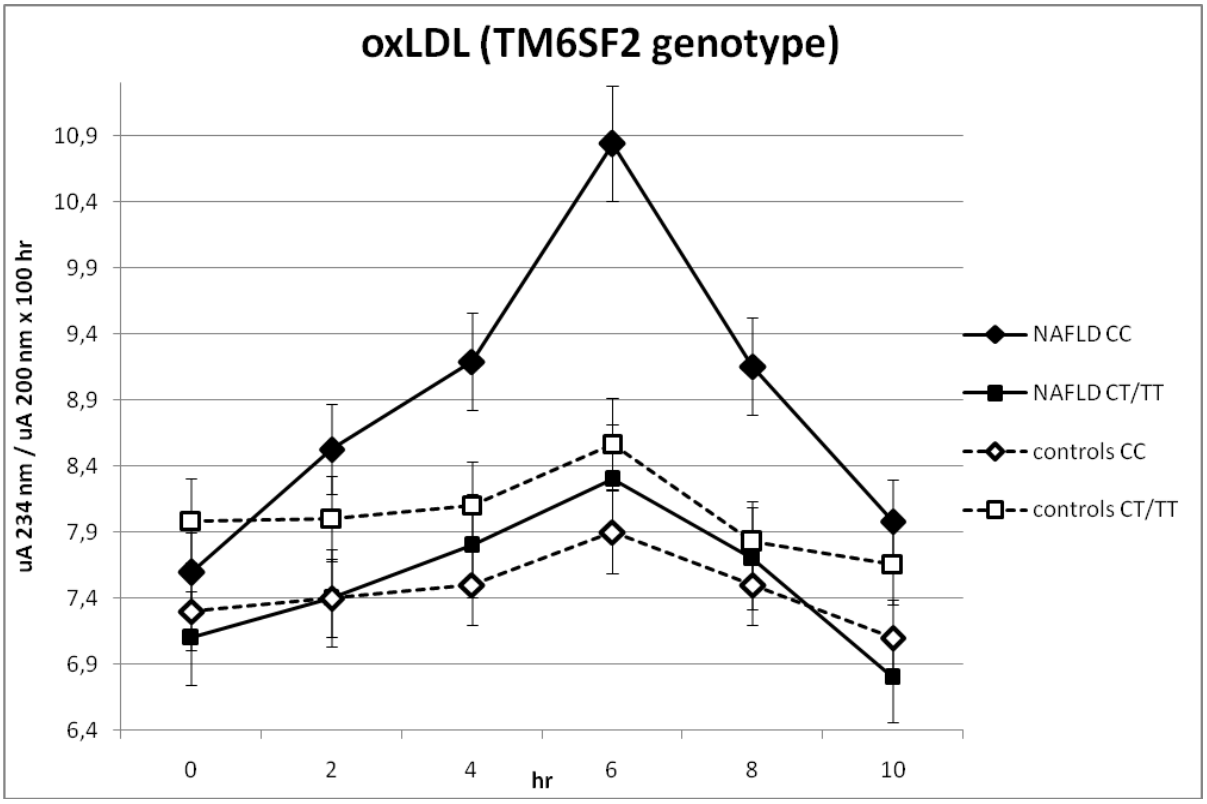
Panel C



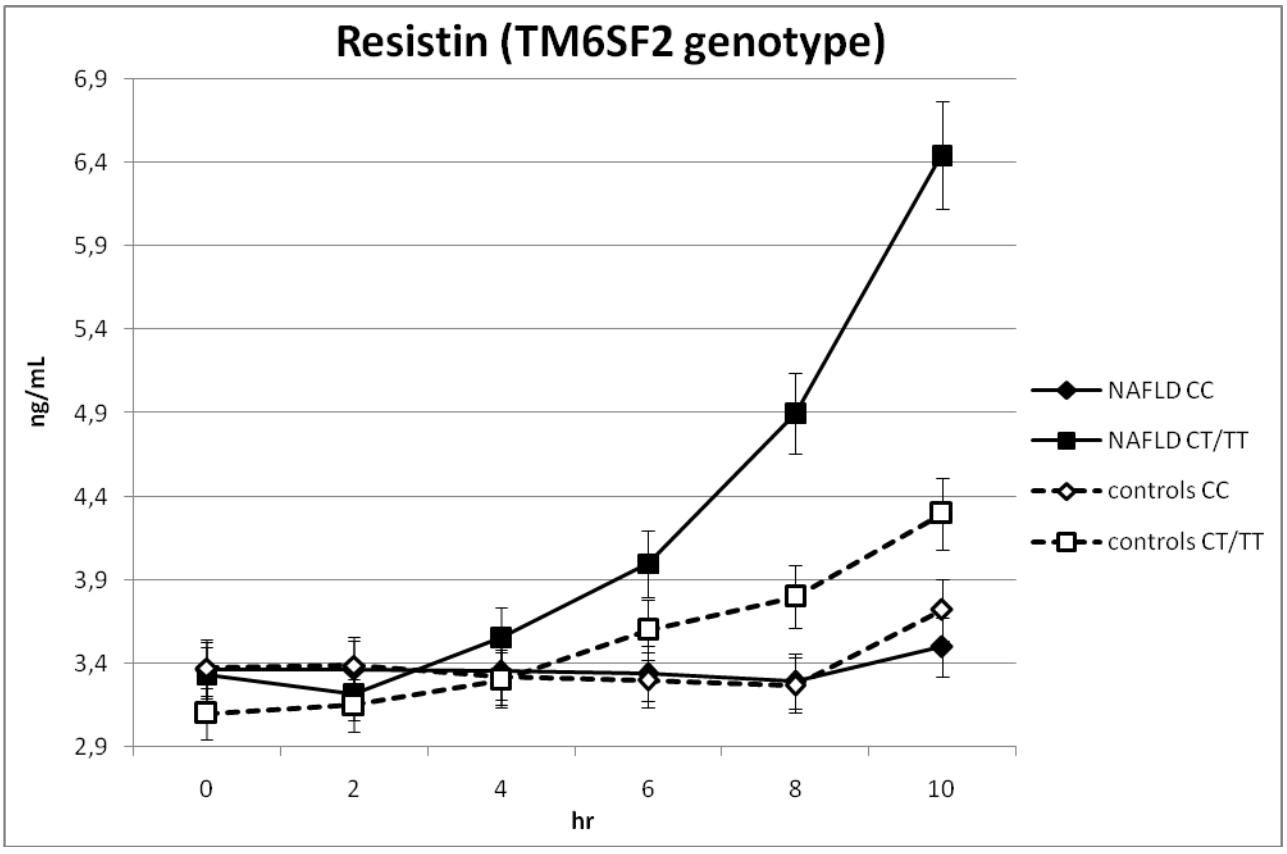
Panel D



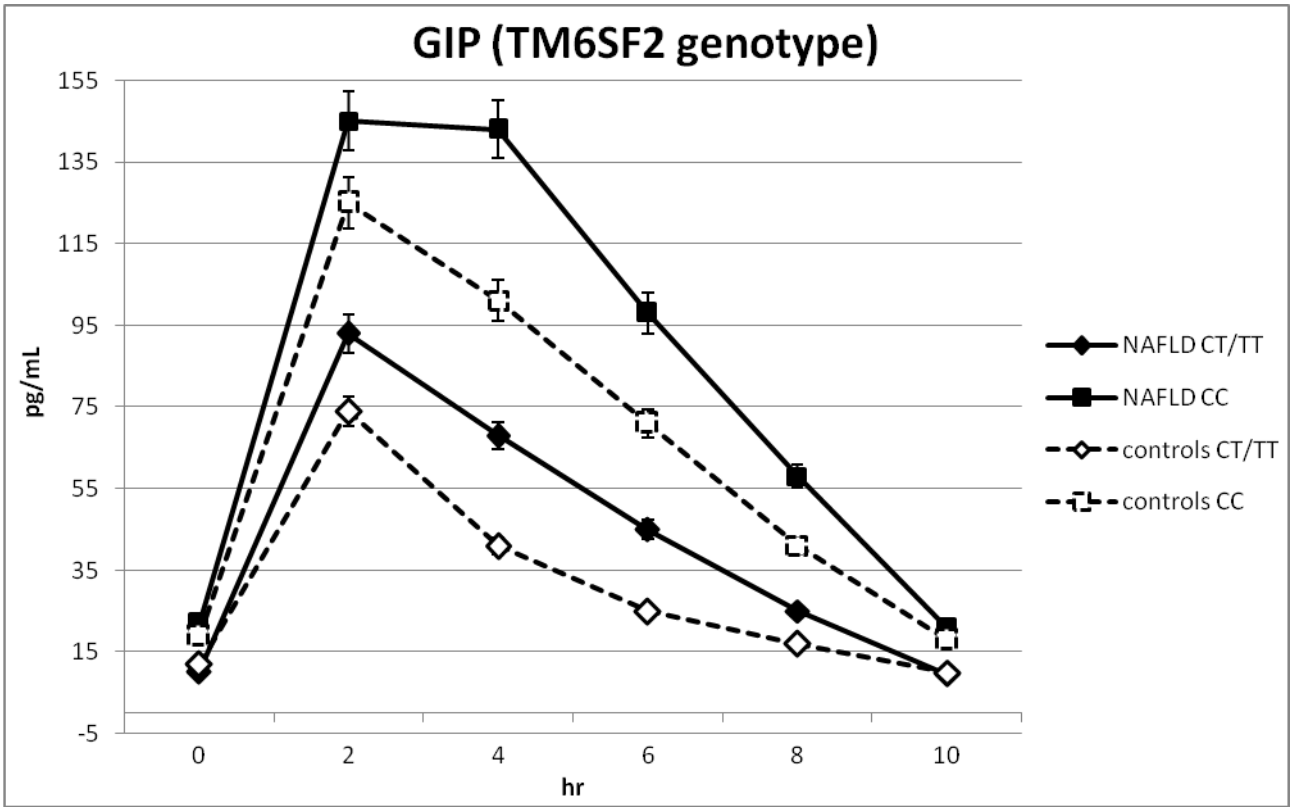
Panel E



Panel F



Panel G


GIP (TM6SF2 genotype)
155
135
115
95
75
55
35
15
-5
pg/mL
0
2
4
6
8
10
hr
NAFLD CT/TT
NAFLD CC
controls CT/TT
controls CC