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Lipoprotein metabolism mediates the association of MTP polymorphism with β-cell dysfunction in healthy subjects and in nondiabetic normolipidemic patients with nonalcoholic steatohepatitis☆

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Abstract

Nonalcoholic steatohepatitis (NASH) predicts incident diabetes independently of insulin resistance, adiposity and metabolic syndrome through unclear mechanisms. Dietary fat consumption and lipoperoxidative stress predispose to diabetes in the general population and to liver injury in NASH. Microsomal triglyceride transfer protein (MTP) polymorphism modulates lipoprotein metabolism in the general population and liver disease in NASH; a functional MTP polymorphism recently predicted incident diabetes independently of insulin resistance in the general population. We simultaneously assessed the impact of MTP polymorphism, diet, adipokines and lipoprotein metabolism, on glucose homeostasis in NASH.

MTP *−493G/T* polymorphism, dietary habits, adipokines and postprandial triglyceride-rich lipoproteins, high-density lipoprotein cholesterol (HDL-C) and oxidized low-density lipoprotein (oxLDL) responses to an oral fat load, were cross-sectionally correlated to oral glucose tolerance test- and frequently sampled intravenous glucose tolerance test-derived Minimal Model indexes of glucose homeostasis in 40 nondiabetic normolipidemic patients with NASH and 40 age-,sex- and body mass index-matched healthy controls.

Despite comparable insulin resistance, fasting lipids, adipokines and dietary habits, MTP GG genotype had significantly more severe β-cell dysfunction; higher plasma Tg, FFA, intestinal and hepatic very low-density lipoprotein 1 subfractions and oxLDL responses and deeper HDL-C fall than GT/TT carriers in patients and controls.

Postprandial HDL-C and oxLDL responses independently predicted β-cell dysfunction and mediated the effect of MTP polymorphism on β-cell function.

In nondiabetic normolipidemic NASH, MTP *−493G/T* polymorphism modulates β-cell function, an effect mediated by postprandial HDL-C and oxLDL metabolism. The impact of this polymorphism on the risk of diabetes and the efficacy of lipid-lowering therapies in restoring β-cell function in NASH, even with normal fasting lipid values, warrant further investigation.

Keywords: Microsomal triglyceride transfer protein; VLDL subfractions; Postprandial; β-cell; Disposition index; ApoB48; Oxidized LDL

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), predisposes to future development of diabetes independently of insulin resistance, adiposity, metabolic syndrome, C-reactive protein and other traditional risk factors [1,2], but genetic and/or environmental mechanism(s) underlying this association are unclear; which subjects are at greater risk of developing diabetes and should therefore be more aggressively treated is not known. Nonalcoholic steatohepatitis (NASH) is the progressive form of NAFLD and a major risk factor for liver-related complications. Different therapeutic approaches (i.e., weight loss, insulin sensitizers, hypolipemizing drugs, antioxidants) targeting the most prominent accompanying metabolic disorder have been proposed for treatment of NASH: recently, incretin mimetics, a class of drugs used to improve β-cell dysfunction in diabetes, showed promising preliminary results [3].Oxidative stress plays a crucial role in the progression of liver injury in NASH and is believed to play a role in the deterioration of glucose homeostasis characterizing diabetes [4,5]. In particular, lipoperoxidative products, including oxidized low-density lipoprotein (oxLDL), triggered the necroinflammatory and fibrogenic process in experimental NASH and correlated with the severity of liver histology in subjects with NASH [6]. Growing evidence connects altered lipoprotein metabolism and oxLDL particle accumulation to the deterioration of β-cell function leading to diabetes [4,7,8]. The incubation of human pancreatic islets with oxLDL particles led to β-cell dysfunction through c-Jun N-terminal

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Table 1
Baseline characteristics of controls and patients with NASH

	Controls			NASH		
	GG (n=20)	GT/TT (n=20)	P	GG (n=20)	GT/TT (n=20)	P
Age (years)	43±2	41±3	.498	42±2	44±2	.698
Sex (% male)	70	68	.679	70	67	.779
Smokers (%)	36	38	.667	32	34	.598
BMI (kg/m^2)	25.1±0.5	25.2±0.6	.458	25.3±0.6	25.1±0.5	.454
% Body fat	23±3	22±2	.601	22±2	23±2	.890
Waist (cm)	90±3	90±3	.740	91±2	90±2	.589
Waist-on-hip ratio	0.91±0.03	0.91±0.03	.902	0.93±0.02	0.92±0.03	.481
Systolic BP (mmHg)	128±3	127±3	.409	128±3	125±2	.514
Diastolic BP (mmHg)	78±1	77±1	.871	87±2[§,‡]	87±2[§,‡]	.989
Total C (mmol/L)	4.56±0.18	7.38±0.16	.340	4.61±0.21	4.64±0.21	.845
LDL-C (mmol/L)	2.69±0.18	2.64±0.13	.326	2.90±0.21	2.85±0.18	.616
Glucose (mmol/L)	5.05±0.22	4.99±0.22	.391	5.22±0.22	5.16±0.17	.261
Insulin (pmol/L)	31.9±13.9	29.2±13.2	.308	93.1±13.9	84.0±10.4[§,‡]	.629
AST (U/L)	14±2	13±3	.567	34±3[§,‡]	32±4[§,‡]	.697
ALT (U/L)	16±3	18±3	.597	81±6[§,‡]	62±7[§,‡]	.126
TNF-α (pg/ml)	1.21±0.11	1.09±0.08	.379	1.14±0.09	1.33±0.09	.457
Adiponectin (ng/ml)	10784±1023	9850±940	.291	5058±506[§,‡]	5928±611[§,‡]	.694
Leptin (pg/ml)	2184±845	2208±786	.301	1605±183	1618±219	.875
Resistin (ng/ml)	3.93±0.42	4.11±0.36	.299	4.24±0.31	3.82±0.86	.692
ApoE genotype (% subjects)						
2–3	16	14	.676	12	13	.589
3–3	70	68	.958	67	69	.972
3–4	14	18	.569	21	18	.890
Abdominal obesity (% subjects)	28	32	.457	35	31	.573
IGR (% subjects)	10	7	.308	29[*,†]	34[*,†]	.689
Hypertension (% subjects)	49	53	.211	68[*]	72[*]	.513
Low HDL-C (%)	9	5	.201	27[*,†]	12[†]	.211
High Tg (% subjects)	4	6	.123	14	6	.287
Subjects with MS (%)	6	5	.345	51[*,†]	44[*,†]	.679
Steatosis (% hepatocytes)	–	–	–	46±10	26±5	.002
Necroinflammatory grade	–	–	–	2.3±1.0	1.4±0.2	.007
Fibrosis stage	–	–	–	2.4±1.0	1.1±0.6	.006

Data are presented as mean±S.E.M.
BP, blood pressure; total C, total cholesterol; LDL-C, LDL cholesterol; WHR, waist-on-hip ratio; IGR, impaired glucose regulation; TNF-α, tumor necrosis factor-α; MS, metabolic syndrome.
[*] P<.05 vs. controls GG.
[†] P<.01 vs. controls GT/TT.
[§] P<.01 vs. controls GG.
[‡] P<.01 vs. controls GT/TT.

kinase (JNK) pathway activation, an effect prevented by coincubation with high-density lipoproteins (HDL) [9].

Genetic or acquired mechanisms underlying the increased oxidative stress in these subjects are under investigation; furthermore, it is unclear if the same mechanisms predisposing to liver disease can also affect β-cell function in NASH, thus explaining the increased metabolic risk of these subjects.

Among environmental factors, wrong dietary habits, in particular fat excess and antioxidant deficiency, have been linked to both NASH and diabetes [5,10]. Among genetic factors, the functional polymorphism *−493G/T* in the microsomal triglyceride transfer protein (MTP) gene promoter has been linked to the presence and severity of liver disease and to impaired lipoprotein metabolism in NASH: GG homozygosis, carrying a lower MTP activity, is a risk factor for liver disease and predisposes to postprandial lipoprotein accumulation and more severe liver injury in NASH [11,12].

Functional MTP polymorphisms affect lipid and lipoprotein levels in the general population and have been recently associated with incident diabetes in a large study [13,14]; interestingly, in this cohort, MTP was associated with insulin response to glucose during an oral glucose tolerance test (OGTT), thus suggesting MTP polymorphisms may modulate glucose homeostasis through mechanisms so far unknown.

We hypothesized that MTP polymorphism may modulate glucose homeostasis in NASH and contribute to the increased risk of diabetes of this population and that this relationship was at least in part mediated by impaired postprandial lipoprotein metabolism and oxLDL accumulation.

The impact of *MTP −493G/T* polymorphism on parameters of glucose homeostasis and the interaction of postprandial lipoprotein metabolism, dietary habits, circulating adipokines with parameters of glucose homeostasis were assessed in non-obese non-diabetic normolipidemic patients with biopsy-proven NASH. Obese and diabetic subjects were excluded, since we aimed at identifying *early* mechanisms predisposing to cardio-metabolic disease and different adipokines may intervene as metabolic disease progresses to diabetes, dyslipidemia and obesity. Furthermore, obesity is *per se* associated with β-cell dysfunction [15]. Lipoprotein metabolism was assessed in the postprandial phase because postprandial lipemia is an established cardiovascular risk factor and contributes substantially to liver triglyceride (Tg) accumulation in NAFLD and abnormal postprandial lipoprotein metabolism is not readily accessible in routine clinical practice [16].

2. Materials and methods

2.1. Patient selection

Based on previous studies on NASH subjects and on *MTP −493G/T* polymorphism [11,12], we considered a Type I error of 0.05 and a Type II error of 0.20: at least 18 NASH subjects per arm were needed to detect a significant between-genotype difference in parameters of glucose homeostasis.

Forty patients and 40 age-, sex-, body mass index (BMI)- and waist-matched healthy controls, partly enrolled in a previous study [12], were selected according to the following criteria: persistently (>6 months) elevated liver enzymes and ultrasono-

graphic bright liver with no liver or biliary tract disease (Table 1). Exclusion criteria were a history of alcohol consumption >40 g/wk (detailed interview extended to family members and by a validated questionnaire filled in daily for 1 week by the patients); BMI >30 kg/m^2; positive markers of viral, autoimmune hepatic disease or celiac disease; abnormal copper metabolism, thyroid function or serum α1-antitripsin levels; overt dyslipidemia (fasting serum cholesterol ≥200 mg/dl or plasma triglyceride ≥200 mg/dl) or diabetes and exposure to occupational hepatotoxins or drugs known to be steatogenic, hepatotoxic or to affect lipid/glucose metabolism.

MTP −493G/T gene polymorphism was assessed by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) using a two-step nested PCR. Mutations in the hemochromatosis genes *HFE* and *TRF2* were detected in patients and controls using multiplex amplification reaction (Nuclear Laser Medicine, Milan, Italy). All patients had a histological diagnosis of NASH, as proposed by Brunt [17].

To further rule out mild fatty liver in controls, beside a negligible alcohol intake (<20 g/day in men and <10 g/day in women) and normal abdomen ultrasound, upper healthy limit for ALT levels was lowered to <30 U/L for men and <20 U/L for women, to increase negative predictive value of a normal result [18,19].

Patients gave their consent to the study, which was conducted according to the Helsinki Declaration.

2.2. Alimentary record

Patients filled in daily a 1-week dietary record according to the European Prospective Investigation into Cancer and Nutrition (EPIC) protocol, which was analyzed using the WinFood database (Medimatica, TE Teramo, Italy) [20,21].

2.3. Cytokines

Serum tumor necrosis factor-α, human adiponectin and leptin were measured by sandwich enzyme-linked immunosorbent assays (R&D System Europe, Abingdon, UK). The sensitivities and coefficients of variation of each assay are reported in online Appendix.

2.4. OGTT- and FSIGTT-derived minimal model indexes of glucose homeostasis

After completion of the alimentary record, patients underwent a standard 75-g OGTT and a frequently sampled intravenous glucose tolerance test (FSIGTT) at 1-week interval and parameters of glucose homeostasis were calculated.

2.4.1. OGTT

Areas under the concentration curves (AUC) of glucose, insulin and C-peptide during the OGTT were calculated with the trapezoidal method. Prehepatic insulin delivery was estimated as the suprabasal (Δ) 30-min AUC of C-peptide divided by the 30-min increase in circulating glucose. Insulin sensitivity was estimated from a model of glucose clearance, which provides OGTT-derived insulin sensitivity (OGIS), an index of insulin sensitivity which has been validated against clamp in nondiabetic subjects [22]. The conventional Quantitative Insulin Sensitivity Check Index (QUICKI) was also determined from the fasting levels of glucose and insulin. The hepatic insulin extraction (He), as percentage of secreted hormone, was estimated by [1−(AUC insulin/AUC C-pep)].

Two OGTT-derived indexes of β-cell function, the insulinogenic index (IGI), computed as the suprabasal serum insulin increment divided by the corresponding plasma glucose increment in the first 30 min (ΔI30/ΔG30) which is an accurate predictor of future development of diabetes [23], and the CP-genic index (CGI), computed as ΔC-pep30/ΔG30, previously validated against measures of β-cell functions derived from frequently sampled intravenous glucose tolerance test [24], were calculated.

β-Cell ability to adapt insulin secretion to changes in insulin sensitivity was assessed by two indexes, the disposition index (DI) and the adaptation index (AI), calculated by multiplying insulin sensitivity indexes (OGIS, QUICKI) times IGI and CGI, respectively. These indexes relate β-cell insulin secretion to insulin resistance and represent integrated parameters of β-cell function, validated against FSIGTT Minimal Model parameters in nondiabetic subjects [25].

2.4.2. Frequently sampled intravenous glucose tolerance test

After an overnight fast, an intravenous glucose tolerance test (0.3 g/kg body weight glucose bolus administered at time zero) was performed. Blood samples were collected in the following 3 h for glucose, insulin and C-peptide concentration measurements and data were analyzed by Minimal Model technique to yield the following parameters of glucose homeostasis: insulin sensitivity index (S_I), acute insulin response to glucose bolus (AIR_G), β-cell sensitivities to glucose (FI1c and FI2c) and average hepatic insulin extraction (Hmean). Two indexes were calculated, which relate β cell insulin secretion and posthepatic delivery to insulin resistance: DI calculated by multiplying S_I times AIR_G and AI, calculated by multiplying S_I times ϕ_1. For a detailed description of these indexes, see online Appendix.

2.4.3. Oral fat load

Patients and controls underwent an oral fat load test, as follows. They were encouraged to avoid strenuous physical efforts and to follow their usual diet during the 24 h preceding the test. The fat load consisted of a mixture of dairy cream (35% fat) and egg yolk (total energy: 766 kcal; fat content: 78.3 g, 65.6% saturated fatty acids, 29.9% monounsaturated fatty acids, 4.5% polyunsaturated fatty acids). The fat load was consumed during a period of 5 min; subjects kept fasting on the test morning, and strenuous activity was forbidden, since exercise can reduce postprandial lipemia. A catheter inserted in the antecubital vein and kept patent during the test was used to draw samples for biochemical determinations. Samples were drawn at 0 (baseline), +2, +4, +6, +8 and +10 h. Plasma total cholesterol, Tg and free fatty acids (FFA) were measured by automated enzymatic methods. ApoE genotype was determined by PCR amplification of genomic DNA using specific oligonucleotide primers.

2.5. Triglyceride-rich lipoprotein subfractionation and measurement of low-density lipoprotein lipid peroxidation

Triglyceride-rich lipoproteins were subfractionated, and low-density lipoprotein (LDL) conjugated diene content was assessed, as a marker of LDL lipoperoxidation, as detailed in online Appendix.

2.6. Statistical analysis

Data were expressed as mean±S.E.M. Differences were considered statistically significant at $P<.05$.

Differences between groups were analyzed by analysis of variance (ANOVA) when variables were normally distributed; otherwise, the Kruskal–Wallis test was used. Normality was evaluated by Shapiro–Wilk test. Chi-square test or Fisher's Exact test were used to compare categorical variables.

Data from the oral fat load were compared by ANOVA and Scheffè post hoc test after log normalization of skewed variables.

The AUC and incremental area under the curve AUC (IAUC) of plasma Tg FFA and conjugated dienes during the oral fat load were computed by the trapezoid method.

MTP polymorphisms were modeled as an additive effect, that is, quantitative predictor variables reflecting the number of risk alleles (0, 1 or 2) as defined previously [26]. Univariate correlations of genetic polymorphisms, dietary, anthropometric and metabolic parameters were made using nonparametric Spearman rank test. Discrete variables were divided into classes for analysis. When a relation was found on univariate analysis, multiple regression analyses were used to estimate relationship between different variables after log transformation of skewed data.

The hypothesis that postprandial oxLDL and high-density lipoprotein cholesterol (HDL-C) responses may mediate the relationship of MTP polymorphism, and β-cell function was then tested. Mediator was defined as a variable hypothesized to lie on the causal pathway between MTP polymorphism and β-cell dysfunction and was assessed in the following five steps [27]:

1. Determine if the genetic trait (MTP polymorphism) predicts the mediator (postprandial oxLDL, postprandial HDL response).
2. Determine if the mediator independently predicts the outcome (β-cell dysfunction).
3. Determine the independent association of β-cell dysfunction with MTP polymorphism *without* including oxLDL and HDL-C responses. This β coefficient represents the "global effect" of MTP polymorphism. A regression model was used to identify independent predictors for β-cell function, as separately assessed by dynamic DI and dynamic AI. The covariates were MTP polymorphism, adiponectin, fasting HDL-C, IAUC-FFA and IAUC-very low-density lipoprotein (VLDL) 1 ApoB48.
4. Determine the association of β-cell dysfunction with MTP polymorphism *with* oxLDL and HDL-C responses in the regression model. This represents the "direct effect" of MTP polymorphism without the effect mediated by oxLDL and HDL-C responses.
5. Calculate mediation, i.e., the change in the β coefficient of MTP polymorphism after adjustment for oxLDL and HDL-C responses. It was calculated as follows: (coefficient for global effect−coefficient for direct effect)/coefficient for global effect×100. Statistical significance of mediation was assessed by Sobel test.

3. Results

3.1. Subjects characteristics

Main features of patients with NASH grouped according to *MTP −493G/T* genotype are reported in Table 1. Due to their low prevalence and to the comparable features, homozygous TT carriers were grouped together with heterozygous GT carriers.

There was no difference between GG and GT/TT genotype in age, sex, overall and abdominal adiposity, fasting glucose, insulin, triglycerides, total and LDL cholesterol. There was no significant difference in ApoE allelic frequency between each of the four groups. Patients with NASH had a higher prevalence of metabolic syndrome as defined by Adult Treatment Panel (ATP) III criteria, but there was no

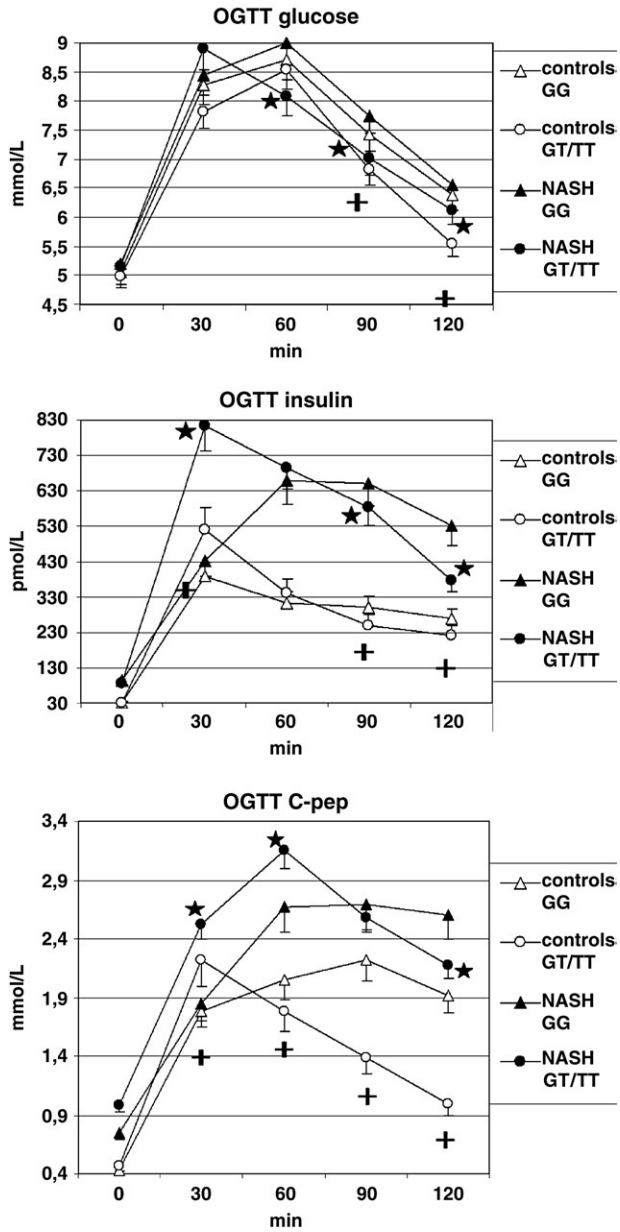

Fig. 1. Time-point curves of plasma glucose, insulin and C-peptide during the OGTT. *P<.05 vs. NASH GG genotype; †P<.05 vs. controls GG genotype.

difference in any single feature or overall presence of metabolic syndrome (at least three criteria met) between MTP genotypes, with 51% NASH GG and 44% NASH GT/TT displaying the whole picture of the metabolic syndrome (at least three criteria met). Patients with NASH had a more severe liver histology than their counterpart GT/TT genotype, as previously reported [12].

3.2. Alimentary record

There was no difference in daily total energy, macro- and micronutrient intake between NASH and controls: total calories: 2493±101 vs. 2509±113 kcal/day (P=.874); carbohydrate: 48±2 vs. 49±3% kcal/day (P=.762); protein: 18±2 vs. 21±2% kcal/day (P=.402); fat: 34±2 vs. 34±2% kcal/day (P=.974); saturated fat: 13.4±0.7 vs. 13.8±0.9% kcal/day (P=.882); polyunsaturated fat (PUFA): 3.6±0.4 vs. 3.7±0.4% kcal (P=.790); monounsaturated fat: 17.4±0.6 vs. 16.9±0.8% kcal (P=.737); ethanol intake: 11±2 vs. 9±3 g/day (P=.781). There was no difference between daily total energy, macro- and micronutrient intake between GG and GT/TT genotype in either NASH or controls (not shown).

3.3. OGTT Minimal Model indexes of glucose homeostasis

Time course of plasma glucose, serum insulin and C-peptide during the OGTT is represented in Fig. 1. OGTT-derived indexes of glucose homeostasis are reported in Table 2. Patients with NASH were insulin resistant and displayed β-cell dysfunction compared to healthy controls, while hepatic insulin extraction was similar between the two groups. In both NASH and controls, MTP GG carriers had more severe β-cell dysfunction than GT/TT genotype, while indexes of insulin sensitivity and hepatic insulin extraction were comparable between MTP genotypes. Basal and dynamic DI and AI were significantly lower in MTP GG carriers than in their counterpart genotypes in both patients and controls.

3.4. FSIGTT Minimal Model parameters

NASH patients showed a lower S_I than controls (Table 2). First-phase β-cell sensitivity to glucose (FI1c) and AIR_G were significantly lower in NASH MTP GG carriers than in other NASH MTP genotypes or in controls. Both DI and AI were significantly lower in MTP GG carriers than in their counterpart genotypes in patients and controls.

3.5. Oral fat tolerance test

MTP GG homozygotes had a significantly higher postprandial plasma Tg, FFA and LDL conjugated diene responses than their counterpart GT/TT in both patients and controls (Table 3). Fasting and postprandial HDL-C was lower in GG than in GT/TT carriers in both NASH and controls.

Postprandial intestinal and hepaticVLDL1 responses were higher in homozygous GG than in the other genotypes, while smaller VLDL2 responses were comparable between different MTP genotypes (Table 3).

LDL-C did not significantly change throughout the test (not shown).

3.6. Correlative analysis

Main results of correlative analysis are reported in Table 4.

QUICKI correlated with BMI waist, OGIS, adiponectin. OGIS correlated with BMI, waist, QUICKI, dynamic (dyn) AI, adiponectin. IGI correlated with MTP polymorphism, dyn DI, CGI, dyn AI, dietary PUFA intake, IAUC LDL conjugated dienes, IAUC HDL-C and adiponectin.

CGI correlated with MTP polymorphism, QUICKI, IGI, dyn AI, IAUC FFA, dietary PUFA intake, IAUC LDL conjugated dienes, fasting and postprandial HDL-C and adiponectin.

DYN DI correlated with MTP polymorphism, OGIS, IGI, CGI, dyn AI, IAUC FFA, IAUC LDL conjugated dienes, IAUC VLDL1 ApoB48, fasting and postprandial HDL-C and adiponectin.

DYN AI correlated with MTP polymorphism, OGIS, IGI, CGI and dyn AI, IAUC FFA, IAUC LDL conjugated dienes, IAUC VLDL1 ApoB100, IAUC HDL-C.

OGTT-derived minimal model parameters were in good correlation with FSIGTT-derived model parameters of glucose homeostasis. Spearman coefficients between FSIGTT-derived and OGTT-derived parameters are reported hereafter.

FSIGTT-derived S_I correlated with OGTT-derived parameters QUICKI (r_s=0.62; P=.0003) and OGIS (r_s=0.75; P=.00004).

AIR_G correlated with IGI (r_s=0.73; P=.00009).

FI1c correlated with CGI (r_s=0.60; P=.0005).

Disposition Index correlated with basal (r_s=0.60; P=.0005) and dynamic DI (r_s=0.65; P=.0001). Adaptation Index correlated with basal (r_s=0.58; P=.0003)and dynamic AI (r_s=0.62; P=.0002).

Table 2
OGTT- and FSIGTT-derived Minimal Model indexes of glucose homeostasis of patients with NASH according to *MTP −493G/T* polymorphism (mean±S.E.M.)

OGTT-derived Minimal Model parameters

	Controls			NASH		
	GG (n=20)	GT/TT (n=20)	P	GG (n=20)	GT/TT (n=20)	P
QUICKI	0.38±0.02	0.39±0.02	.751	0.31±0.02*,†	0.32±0.02*,†	.672
OGIS	456.1±15.3	461.2±18.4	.587	381.9±14.1*,†	383.2±16.4*,†	.532
He	75±5	75±6	.891	72±4	74±5	.978
IGI	101.4±11.5	199.9±21.0	.0004	59.4±12.1*,‡	138.6±18.9†	.0004
CGI	475.4±38.6	605.9±46.8	.008	382.6±16.1§,‡	548.1±35.2†	.001
Basal DI	43.5±4.2	76.9±8.9	.0007	19.9±3.5§,‡	44.3±7.8‡	.00009
Dyn DI	45798±4112	85202±8731	.0001	37113±3176§,‡	68571±8803*,†	.0001
Basal AI	185.1±18.3	245.2±16.3	.009	121.3±14.8§,‡	174.3±17.1†	.002
Dyn AI	217215±41012	279402±43791	.002	145012±17102§,‡	227891±42113†	.0004

FSIVGTT-derived Minimal Model parameters

	Controls			NASH		
	GG	GT/TT	P	GG	GT/TT	P
S_I	7.43±0.90	8.01±1.11	.873	3.52±0.41§,‡	3.12±0.61§,‡	.712
FI1c	96.4±18.7	138.4±19.4	.002	62.1±12.2§,‡	94.0±19.2†	.009
AIR_G	94.3±11.2	134.7±12.1	.001	60.3±8.3§,‡	101.3±11.3†	.007
DI	661.7±34.5	1081.5±69.7	.0008	227.0±23.2§,‡	319.1±46.4§,‡	.005
AI	678.7±74.7	1116±83.1	.0002	253.8±34.3§,‡	302.2±43.0§,‡	.008
Hmean	73±4	74±4	.214	73±5	72±6	.896

Units of the main parameters presented: OGIS, oral glucose tolerance test-derived insulin sensitivity; ml min^{-1} m^{-2}); He, (%); IGI, (Δi30/Δg30; $\mu U_{insulin}$ $g^{-1}{}_{glucose}$); CGI, (Δcp30/Δg30; $ng_{\bar{C}\,pep}$ $g^{-1}{}_{glucose}$); Basal DI, (QUICKI×IGI; $\mu U_{insulin}$ $g^{-1}{}_{glucose}$); Dyn DI, (OGIS×IGI; $\mu U_{insulin}$ $g^{-1}{}_{glucose}$ ml^{-1} m^{-2}); Basal AI, (QUICKI×CGI; $ng_{\bar{C}\,pep}$ $g^{-1}{}_{glucose}$); Dyn AI, (OGIS×CGI; $ng_{C\text{-}pep}$ $g^{-1}{}_{glucose}$ ml^{-1} m^{-2}); S_I, (10^{-4} min^{-1} μU^{-1} ml^{-1}); FI1c, (pmol $min^{-1}mg^{-1}dl$); AIR_G, (μU/ml); DI, (min^{-1}); AI, (min^{-2} $mmol^{-1}$ L); Hmean, (%).

* P<.05 vs. controls GG.
† P<.05 vs. controls GT/TT.
§ P<.01 vs. controls GG.
‡ P<.01 vs. controls GT/TT.

FSIGTT-derived indexes correlated with different metabolic parameters similarly to corresponding OGTT-derived parameters (not shown).

3.7. Assessment of postprandial oxLDL and HDL-C responses as mediating variables

The five-step analysis of the role of postprandial oxLDL and HDL-C in mediating the association of MTP polymorphism with β-cell dysfunction is reported below.

Step I: MTP polymorphism predicts postprandial oxLDL and HDL responses. *−493G/T* MTP was an independent predictor of IAUC LDL conjugated diene (β=0.52; SEβ=0.14; P=.001) and IAUC HDL-C (β=−0.50; SE_β=0.15; P=.003).

Step II: oxLDL and HDL responses independently predict β-cell dysfunction.

IAUC LDL conjugated dienes independently predicted dyn DI (β=−0.50; SE_β=0.13; P=.004) and dyn AI (β=−0.49; SE_β=0.16; P=.008). Similarly, IAUC HDL-C independently predicted dyn DI (β=0.49; SE_β=0.15; P=.009) and dyn AI (β=0.47; SE_β=0.18; P=.01).

Table 3
Oral fat load parameters of patients with NASH and controls according to *MTP −493G/T* polymorphism

	Controls			NASH		
	GG (n=20)	GT/TT (n=20)	P	GG (n=20)	GT/TT (n=20)	P
Fasting Tg (mmol/L)	0.88±0.08	0.90±0.11	.597	0.96±0.12	1.06±0.14*	.112
IAUC Tg (mmol/L×hr)	1.56±0.52	0.52±0.26	.028	6.64±0.86§,‡	3.29±0.80§,‡	.0001
Fasting VLDL1-ApoB48 (g/L)	0.021±0.004	0.021±0.005	.313	0.024±0.004	0.019±0.003	.718
IAUC VLDL1-ApoB48 (g/L×hr)	0.051±0.015	0.020±0.004	.008	0.125±0.024§,‡	0.078±0.010*,‡	.0007
Fasting VLDL2-ApoB48 (g/L)	0.011±0.004	0.009±0.004	.429	0.014±0.007	0.014±0.005	.668
IAUC VLDL2-ApoB48 (g/L×hr)	0.036±0.024	0.029±0.02	.211	0.061±0.020§,‡	0.083±0.019§,‡	.318
Fasting VLDL1-ApoB100 (g/L)	0.070±0.008	0.061±0.007	.500	0.066±0.009	0.071±0.007	.761
IAUC VLDL1-ApoB100 (g/L×h)	0.142±0.029	0.065±0.021	.001	0.255±0.030§,‡	0.172±0.025§,‡	.0008
Fasting VLDL2-ApoB100 (g/L)	0.069±0.028	0.068±0.028	.222	0.074±0.016	0.070±0.021	.713
IAUC VLDL2-ApoB100 (g/L×h)	0.093±0.039	0.089±0.026	.378	0.101±0.038	0.125±0.029	.499
Fasting FFA (mmol/L)	0.51±0.11	0.46±0.19	.313	0.89±0.35*,†	0.85±0.19*,†	.566
IAUC FFA (mmol/L×h)	0.70±0.32	1.70±0.58	.022	6.09±1.38§,‡	3.16±0.99§,‡	.004
Fasting HDL-C (mmol/L)	1.45±0.05	1.61±0.05	.010	1.17±0.03*,†	1.29±0.03*,†	.002
IAUC HDL (mmol/L×h)	−0.31±0.05	−0.16±0.05	.007	−1.45±0.13§,‡	−0.59±0.10§,‡	.002
Fasting LDL C.D. (μA 234 nm/μA 200 nm×100)	6.91±1.32	6.84±1.39	.232	6.96±1.98	7.32±1.38	.379
IAUC LDL C.D. (μA 234 nm/μA 200 nm×100)×h	2.9±0.4	0.4±0.2	.004	20.3±5.7§,‡	6.6±2.9§,‡	.00009

Data are presented as mean±S.E.M.
C.D., conjugated dienes.

* P<.05 vs. controls GG.
† P<.05 vs. controls GT/TT.
§ P<.01 vs. controls GG.
‡ P<.01 vs. controls GT/TT.

Table 4
Main Spearman correlation coefficients between different variables in patients with NASH

	MTP −493G/T	BMI	Waist	OGIS	QUICKI	IGI	Dyn DI	CGI	Dyn AI	IAUC FFA	IAUC LDL C.D.	IAUC VLDL1 ApoB48	Fasting HDL-C	IAUC HDL-C	IAUC VLDL1 ApoB100	Adiponectin	PUFA, % E
MTP −493G/T	–	0.08	0.02	−0.13	−0.22	**−0.59**‡	**−0.68**‡	**−0.54**†	**−0.60**‡	**0.48**†	**0.62**‡	**0.51**†	**−0.42***	**−0.57**‡	**0.50**†	−0.31	0.02
BMI	0.08	–	**0.54**‡	**0.52**†	**0.51**†	0.20	0.28	0.20	0.25	0.31	0.03	0.34	0.36	0.27	0.11	**−0.46***	0.02
waist	0.02	**0.54**‡	–	**−0.45***	**−0.44***	−0.23	−0.28	−0.30	−0.35	0.22	0.35	0.14	−0.23	−0.19	0.20	**−0.42***	0.22
OGIS	−0.13	**0.52**†	**−0.45***	–	**0.54**†	0.31	0.38	0.32	**0.41***	−0.30	−0.33	−0.18	−0.38	−0.23	−0.21	**0.47***	0.30
QUICKI	−0.22	**0.51**†	**−0.44***	**0.54**†	–	0.08	0.20	0.03	0.11	0.33	0.21	0.30	0.33	0.20	0.39	**0.41***	0.24
IGI	**−0.59**‡	0.20	−0.23	0.31	0.08	–	**0.68**‡	**0.60**‡	**0.54**†	0.31	**−0.65**‡	−0.39	**0.42***	**0.60**‡	−0.35	**0.46***	0.42*
Dyn DI	**−0.68**‡	0.28	−0.28	**0.42***	0.38	**0.68**‡	–	**0.51**†	**0.62**‡	**−0.43***	**−0.66**‡	**−0.48***	**0.44***	**0.63**‡	−0.29	**0.46***	0.38
CGI	**−0.54**†	0.20	−0.32	0.21	0.18	**0.60**‡	**0.51**†	–	**0.68**‡	**−0.45***	**−0.58**‡	−0.38	**0.46***	**0.54**†	−0.23	**0.45***	0.41*
Dyn AI	**−0.60**‡	0.25	−0.35	**0.41***	0.11	**0.54**†	**0.62**‡	**0.68**‡	–	**−0.45***	**−0.62**‡	−0.36	0.33	**−0.58**‡	**−0.47***	0.36	0.39
IAUC FFA	**0.48***	0.31	0.22	−0.30	0.33	0.31	**−0.43***	**−0.45***	**−0.45***	–	0.39	0.33	0.12	0.31	0.35	**−0.48***	−0.31
IAUC LDL C.D.	**0.62**‡	0.03	0.35	−0.33	0.21	**−0.65**‡	**−0.66**‡	**−0.58**‡	**−0.62**‡	0.39	–	**0.54**†	0.38	**−0.47***	0.39	**−0.44***	−0.34
IAUC VLDL1 ApoB48	**0.51**†	0.34	0.14	−0.18	0.30	−0.39	**−0.48***	−0.38	−0.36	0.33	**0.54**†	–	0.37	0.13	**−0.48***	−0.38	−0.21
Fasting HDL-C	**−0.42***	0.36	−0.23	−0.38	0.33	**0.42***	**0.44***	**0.46***	0.33	0.12	0.38	0.37		**0.49***	0.29	0.39	0.14
IAUC HDL-C	**−0.57**‡	0.27	−0.19	−0.23	0.20	**−0.60**‡	**0.63**‡	**0.54**†	**−0.58**†	0.31	**−0.47***	0.13	**0.49***	–	0.35	0.20	0.22
IAUC VLDL1 ApoB100	**0.50**†	0.11	0.20	−0.21	0.39	−0.35	−0.29	−0.23	**−0.47***	0.35	0.39	**−0.48***	0.29	0.35	–	−0.35	−0.12
Adipo	−0.31	**−0.46***	**−0.42***	**0.47***	**0.41***	**0.46***	**0.46***	**0.45***	0.36	**−0.48***	**−0.44***	**−0.38**	0.39	0.20	−0.35	–	**0.43***
PUFA, % E	0.02	0.02	0.22	0.30	0.24	**0.42***	0.38	**0.41***	0.39	−0.31	−0.34	−0.21	0.14	0.22	−0.12	**0.43***	–

Statistically significant correlations are written in bold characters.
The number of MTP G alleles was seta as one in the correlation (i.e., MTP GG=2; MTP GT=1; MTP TT=0).
IA, incremental area under the curve; Glu, glucose; C, cholesterol; Adipo, adiponectin.
* $P<.05$.
† $P<.01$.
‡ $P<.001$.

Step III: "global effect" of MTP polymorphism on β-cell dysfunction (*without* including oxLDL and HDL-C responses). In two separate multiple regression models adjusted for adiponectin, fasting HDL-C, IAUC-FFA, IAUC-VLDL1 ApoB48, MTP polymorphism independently predicted dyn DI (β=−0.53; SE_β=0.12; P=.0009)and dyn AI (β=−0.52; SE_β=0.13; P=.001).

Step IV: "direct effect" of MTP polymorphism on β-cell dysfunction (including oxLDL and HDL-C responses in the model). After including separately IAUC LDL conjugated dienes and IAUC HDL-C in the model, there was a marked attenuation in β coefficient of β-cell dysfunction from MTP polymorphism. After including both parameters in the model, MTP polymorphism no longer significantly predicted β-cell dysfunction (Table 5).

Step V: calculation of mediation. The magnitude of the attenuation of the relationship between MTP polymorphism and β-cell function indexes is reported in Table 5.

Table 5
Postprandial oxLDL and HDL-C mediation of the effect of *MTP −493G/T* polymorphism on β-cell dysfunction

−493G/T MTP	β (global effect)		β (direct effect)					
		P	IAUC oxLDL	P	IAUC HDL-C	P	Both	P
Dyn DI	−0.53	.0009	−0.35	.03	−0.36	.02	−0.24	.15
Dyn AI	−0.52	.001	−0.34	.03	−0.33	.04	−0.25	.14
			Mediation (%)					
			IAUC oxLDL	P	IAUC HDL-C	P	Both	P
Dyn DI			34	.007*	32	.014*	55	.001*
Dyn AI			35	.02*	37	.039*	52	.009*

β=partial multiple regression coefficient; Global effect, effect of MTP polymorphism on β-cell dysfunction without including oxLDL and HDL-C responses); direct effect, effect" of MTP polymorphism on β-cell dysfunction after including oxLDL response, HDL-C response, or both; Mediation, magnitude of the attenuation of the relationship between MTP polymorphism and β-cell function indexes, calculated as follows: (coefficient for global effect−coefficient for direct effect)/coefficient for global effect×100.
* Statistical significance of mediation was assessed by Sobel test.

4. Discussion

Main findings of our paper are the following:

a) we report here for the first time that MTP *−493G/T* polymorphism is associated with pancreatic β-cell dysfunction, as assessed by two independent and validated methods, in NASH and healthy controls.
b) β-cell dysfunction is associated with the magnitude of postprandial oxLDL accumulation and HDL-C fall in both NASH and controls.
c) postprandial oxLDL and HDL-C responses mediate the association of MTP *−493G/T* polymorphism on β-cell dysfunction.

MTP polymorphism predicts the presence and severity of liver disease in NASH and modulates the lipoprotein phenotype in the general population, but its association with disturbances of glucose homeostasis is unknown. Recently, a large cohort study found an association between a functional MTP polymorphism and incident diabetes [13]. However, mechanism(s) underlying this association are unclear. By correlating MTP *−493G/T* polymorphism to Minimal Model-derived indexes of glucose metabolism, we found homozygous MTP GG carriers have a more severe pancreatic β-cell dysfunction compared to GT/TT genotype in both NASH and healthy controls, despite comparable insulin resistance, adiposity, dietary habits and adipokine levels. Mechanisms connecting MTP polymorphism to pancreatic β-cell dysfunction in the absence of overt fasting hyperlipidemia may include impaired postprandial lipoprotein

metabolism and LDL lipoperoxidation. Postprandially, triglyceride-rich lipoproteins (TRLPs) are hydrolyzed to remnants, LDL are enriched with peroxidation-prone triglyceride and HDLs are depleted of cholesterol via cholesterol-ester transfer protein. The proatherogenic effects of oxLDL and TRLP-remnants on vascular endothelium and macrophages, as well as the protective role of HDL-C, are well documented, and mounting evidence links oxLDL and HDL-C to oxidative injury in the liver [6,12,28]. Epidemiological and experimental evidence suggests oxLDL and HDL-C particles modulate β-cell function. Plasma oxLDL levels predicted incident diabetes over 9 years in the Atherosclerosis Risk in Communities Study [7]. β-Cells uptake VLDL and LDL in lipid-storing vescicles, and coincubation of human oxLDLs dose-dependently enhanced apoptosis and decreased glucose-induced insulin secretion in cultured human β-cells by binding to scavenger receptor and activating the JNK pathway, an effect totally reversed by coincubation of β-cells with HDL-C particles [8,9]. Altogether, these findings suggest impaired lipoprotein metabolism may play an important role in the pathogenesis of diabetes through β-cell dysfunction.

Consistently, postprandial oxLDL and HDL-C responses independently predicted and mediated in our statistical model the effect of MTP polymorphism on β-cell dysfunction in our subjects, thus connecting impaired postprandial lipoprotein metabolism to pancreatic β-cell dysfunction even in the absence of overt hyperlipidemia or diabetes.

Although causality cannot be inferred from our cross-sectional study and prospective confirmation is warranted, our data suggest that lipid intolerance and impaired postprandial lipoprotein metabolism may be an early phenomenon in the pathogenesis of diabetes and may predispose to glucose intolerance in both healthy and NASH subjects. An alternative yet unproven hypothesis is that MTP polymorphism may predispose to pancreatic lipid infiltration by modulating lipid flux and lipoprotein metabolism directly in the β-cell, eventually leading to a nonalcoholic fatty pancreas [29]. Although pancreatic fat infiltration as assessed by magnetic resonance spectroscopy has been found to cross-sectionally correlate with β-cell dysfunction, the presence and functional relevance of MTP in pancreatic β-cells remain to be prospectively elucidated [29]; furthermore, further histological studies need to demonstrate that dysfunctional islets from subjects with nonalcoholic fatty pancreas have increased fatty deposits and/or apoptosis.

Clinical implications of our findings are twofold: first, assessing MTP polymorphism may help identify subjects at higher metabolic risk, who might benefit from therapies targeting not only insulin resistance but also β-cell dysfunction. Second, in at-risk genotypes, therapeutic interventions should target postprandial lipoprotein dysmetabolism even in the presence of normal fasting lipid levels: lipid-lowering drugs are likely to be useful for this purpose, but incretin analogues have also proved effective at modulating postprandial lipid metabolisms [30].

Limitations of this study are its cross-sectional nature, which prevents any causal inference, and the small number of subjects.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2009.06.007.

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