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Polymorphism in Microsomal Triglyceride Transfer Protein: A Link Between Liver Disease and Atherogenic Postprandial Lipid Profile in NASH?

Roberto Gambino,[1] Maurizio Cassader,[1] Gianfranco Pagano,[1] Marilena Durazzo,[1] and Giovanni Musso[2]

Nonalcoholic fatty liver disease (NAFLD) is emerging as an independent cardiovascular risk factor, but mechanism(s) linking fatty liver to atherosclerosis are unknown. Microsomal triglyceride transfer protein (MTP) −493 G/T polymorphism modulates circulating lipid and lipoprotein levels in different subsets and has been linked to NAFLD. The impact of MTP −493 G/T polymorphism, adipokines, and diet on postprandial lipoprotein profile and liver disease was assessed in nonalcoholic steatohepatitis (NASH). Plasma lipids, triglyceride-rich lipoprotein subfractions, high-density lipoprotein-C (HDL-C), and oxidized low-density lipoprotein (LDL) after an oral fat load were cross-sectionally correlated to MTP −493 G/T polymorphism, dietary habits, adipokines, and liver histology in 29 nonobese nondiabetic patients with NASH and 27 healthy controls. The severity of liver histology, the magnitude of triglycerides (Tg), free fatty acid (FFA), and LDL-conjugated diene responses, and the fall in HDL-C and apoA1 were significantly higher in NASH G/G (66% of patients) than in the other genotypes, despite similar adipokine profile and degree of insulin resistance. Postprandial large intestinal very-low-density lipoprotein (VLDL) subfraction A increases independently predicted Tg (β = 0.48; P = .008), FFA (β = 0.47; P = 0.010), HDL-C (β = 0.42; P = 0.009), and LDL-conjugated diene (β = 0.52; P = 0.002) responses. VLDL A apoB48 response was independently associated with liver steatosis (OR: 2.4; CI 1.7-9.6; P = 0.031). Postprandial LDL-conjugated diene response predicted severe necroinflammation (OR: 3.3; CI 1.4-9.7; P = 0.016) and fibrosis (OR: 2.8; CI 1.0-8,5; P = 0.030); postprandial apoA1 fall predicts severe fibrosis (OR: 2.1; CI: 1.5-6.1; P = 0.015). *Conclusion:* MTP -493 G/T polymorphism may impact NASH by modulating postprandial lipemia and lipoprotein metabolism; homozygous GG carriers have a more atherogenic postprandial lipid profile than the other genotypes, independently of adipokines and insulin resistance. (HEPATOLOGY 2007;45:1097-1107.)

Nonalcoholic fatty liver disease (NAFLD), the most common chronic liver disease in Western populations, has been associated with insulin resistance and metabolic syndrome.[1,2] This association prompted research on NAFLD as a cardiovascular risk factor. Surrogate markers of NAFLD predicted incident cardiovascular disease independently of classical risk factors, insulin resistance, and metabolic syndrome, in several large prospective cohort studies.[3,4] Furthermore, liver histology independently correlated with carotid intima-media thickness in NAFLD, suggesting the vessel wall and the liver of these patients share common inflammatory mediators.[5] Mechanism(s) underlying this association are unknown, nor is it known which subjects with nonalcoholic steatohepatitis (NASH) are at greater cardiovascular risk and should therefore be candidates for early aggressive interventions.

The postprandial phase has been linked to atherosclerosis and increased oxidative stress in diabetes.[6] Increased lipid peroxidation may have a role in the pathogenesis of NASH, alone or in association with insulin resistance and impaired adipokine signaling.[1] The liver takes up circu-

Abbreviations: Chol, cholesterol; FFA, free fatty acids; HDL-C, high-density lipoprotein cholesterol; IAUC, incremental area under the postprandial curve; ISI, insulin sensitivity index; LDL, low-density lipoprotein; MTP, microsomal triglyceride transfer protein; NAFLD, non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; Tg, triglyceride; TRLP, triglyceride-rich lipoprotein; VLDL, very-low-density lipoprotein.

From the [1]Department of Internal Medicine, University of Turin, Italy; and [2]Gradenigo Hospital, Turin, Italy.

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Address reprint requests to: Giovanni Musso, Emergency Department, Gradenigo Hospital, C.[50] Regina Margherita 8, 10126 Turin, Italy. E-mail: giovanni_musso@yahoo.it; fax: (39) 11-8151320

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lating free fatty acid (FFA) in a dose-dependent fashion, and low-density lipoprotein (LDL) and remnants through the LDL-receptor and the liver-related receptor protein. Consistently, postprandial lipid storage contributes substantially to the liver triglyceride (Tg) pool in NAFLD[7] and the magnitude of postprandial lipemia predicts liver steatosis.[8,9]

The functional polymorphism −493 G/T in the microsomal triglyceride transfer protein (MTP) gene promoter has been linked to liver disease in NAFLD: GG homozygosity, or carrying a lower MTP activity than the other genotypes, predicted more severe liver histology.[10]

This polymorphism modulates lipid and lipoprotein levels in healthy and hypercholesterolemic subjects,[11,12] but its impact on postprandial lipid metabolism in fatty liver, as well as its interaction with other factors (insulin resistance, adiposity, adipokines, oxidative stress, dietary habits) in the pathogenesis of NASH, are unknown.

The impact of the −493 G/T MTP polymorphism on postprandial plasma lipid metabolism in nonobese nondiabetic patients with NASH and healthy controls and the interaction of this polymorphism with insulin resistance, dietary habits, adipokines, and oxidative stress in determining the severity of liver disease in NASH were assessed.

Patients and Methods

Patient Selection. Twenty-nine patients were selected (Table 1) according to the following criteria: persistently (>6 months) elevated liver enzymes; ultrasonographic presence of bright liver without any other liver or biliary tract disease. Exclusion criteria were: a history of alcohol consumption >40 g/week, as assessed by a detailed interview extended to family members and by a validated questionnaire filled in daily for 1 week by the patients; a body mass index >30 kg/m^2; positive markers of viral, autoimmune, or celiac disease; abnormal copper metabolism, thyroid function or serum α_1-antitrypsin levels; overt dyslipidemia (fasting serum cholesterol ≥200 mg/dl or plasma triglyceride ≥200 mg/dl) or diabetes; exposure to occupational hepatotoxins or drugs known to be steatogenic, hepatotoxic, or to affect lipid/glucose metabolism. Mutations in the hemochromatosis genes HFE and TRF2 were detected in patients and controls using multiplex amplification reaction (Nuclear Laser Medicine, Milan, Italy). Part of the patients participated in previous studies made in our Institution.[9]

All patients had a histological diagnosis of NASH, as proposed by Brunt.[13] Liver iron concentration and hepatic iron index were determined from biopsies by atomic absorption spectroscopy. The controls were 27 healthy subjects comparable for age, sex, body mass index, waist circumference, and waist-on-hip ratio with normal liver enzymes (defined by ALT < 30 U/l in men and < 20 U/l in women, based on the cutoff values provided by Prati et al.,[14] to enhance the negative predictive value of a normal value) and abdomen ultrasound (Table 1).

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

Alimentary Record. Patients and controls filled in daily a dietary record for 1 week, according to the EPIC protocol, analyzed using the WinFood database (Medimatica, TE Teramo, Italy) as described.[9]

Anthropometry. Percent body fat was estimated by the BIA method (TBF-202, Tanita, Tokyo, Japan), closely correlating with dual X-ray absorption measures.[15]

Cytokines. Serum tumor necrosis factor alpha, leptin, and adiponectin were measured by sandwich enzyme-linked immunosorbent assay (R&D System Europe Ltd, Abingdon, UK). Resistin was measured by an enzyme immunoassay (Bio Vendor Laboratory Medicine Inc., Brno, Czech Republic).

Oral Glucose Tolerance Test. After completion of the alimentary record, patients and controls underwent a standard 75-g oral glucose tolerance test. The whole-body insulin sensitivity index (ISI) was calculated.[16]

Oral Fat Load. The 29 patients who had biopsies and 27 matched controls underwent an oral fat load test, as previously described.[17] Samples were drawn at 2-hour intervals for 10 hours. Plasma total cholesterol (Chol), triglyceride (Tg) and free fatty acids (FFA) were measured by automated enzymatic methods. Apo E genotype was determined by PCR amplification of genomic DNA using specific oligonucleotide primers. The −493 G/T MTP gene polymorphism was assessed by PCR- **restriction fragment length polymorphism** using a two-step nested PCR.

Separation of Triglyceride-Rich Lipoprotein Subfractions. Very-low-density lipoproteins (VLDL) were isolated through preparative ultracentrifugation and subsequently assayed for their Tg and total Chol content. One aliquot plasma was brought to densities of 1,006 g/l by adding a KBr solution (d = 1,330 g/l) and centrifuged at 105,000*g* for 21 hours at 10°C in a Beckman L8-70M ultracentrifuge. Tg and total Chol concentration was then determined in lipoprotein fraction enzymatically. The first higher amount of blood (10 ml) was drawn for subfractionating **triglyceride-rich lipoprotein** (TRLPs) by ultracentrifugation on a discontinuous density gradient. Separated plasma was brought to a density of 1.10 g/ml by adding solid KBr. The density gradient was prepared by adding to 4 ml of this plasma 3 ml of a 1.065 g/ml solu-

Table 1. Baseline Characteristics of Controls and Patients With NASH According to -493 G/T MTP Polymorphism

	Controls GG (n = 13)	Controls GT/TT (n = 14)	*P* Controls	NASH-GG (n = 19)	NASH-GT/TT (n = 10)	*P* NASH
Age (years)	35 ± 2	37 ± 2	0.890	39 ± 2	37 ± 3	0.597
Sex (M/F)	12/2	11/3	0.493	16/3	8/2	0.749
Smokers (%)	31	35	0.596	28	32	0.581
% Body fat	22 ± 3	23 ± 3	0.896	23 ± 3	23 ± 2	0.714
BMI (kg/m^2)	25.0 ± 0.7	25.1 ± 0.8	0.947	24.9 ± 0.5	25.6 ± 0.9	0.533
Waist (cm)	88 ± 2	90 ± 2	0.750	90 ± 2	91 ± 3	0.681
WHR	0.91 ± 0.02	0.92 ± 0.03	0.697	0.93 ± 0.02	0.92 ± 0.01	0.593
Systolic BP (mmHg)	124 ± 2	125 ± 2	0.957	127 ± 3	125 ± 3	0.519
Diastolic BP (mmHg)	79 ± 2	78 ± 2	0.795	87 ± 2‡§	87 ± 2‡§	0.956
Triglycerides (mg/dl)*	62 ± 5	79 ± 6	0.041	78 ± 7	104 ± 9*†	0.034
Total C (mg/dl)†	173 ± 7	181 ± 8	0.496	182 ± 10	179 ± 9	0.187
HDL-C (mg/dl)†	61 ± 1	59 ± 1	0.897	45 ± 1‡§	50 ± 2†§	0.004
HDL2-C (mg/dl)	20 ± 2	19 ± 2	0.495	11 ± 1‡§	19 ± 2§	0.006
HDL3-C (mg/dl)	41 ± 1	40 ± 1	0.139	34 ± 2‡§	31 ± 2‡§	0.622
LDL-C (mg/dl)†	101 ± 6	108 ± 7	0.567	116 ± 10	110 ± 8	0.759
ApoB (mg/dl)	82 ± 6	83 ± 6	0.495	84 ± 5	86 ± 5	0.814
Glucose (mg/dl)	91 ± 2	89 ± 2	0.768	94 ± 2	101 ± 4	0.783
Insulin (μU/mL)	4.2 ± 1.5	3.8 ± 1.4	0.586	13.5 ± 2.2‡§	12.6 ± 1.6‡§	0.814
Whole body ISI	8.01 ± 0.81	7.38 ± 0.62	0.549	4.59 ± 0.78‡§	3.52 ± 0.81‡§	0.284
AST (U/l)	24 ± 3	19 ± 2	0.697	46 ± 4‡§	38 ± 4‡§	0.231
ALT (U/l)	22 ± 4	21 ± 5	0.792	92 ± 4‡§	75 ± 5‡§	0.0258
GGT (U/l)	41 ± 5	34 ± 5	0.769	116 ± 15‡§	65 ± 14‡§	0.042
Total Br (mg/dl)	0.9 ± 0.3	0.8 ± 0.2	0.998	1.1 ± 0.3	1.0 ± 0.4	0.784
TNF-$\alpha\alpha$ (pg/ml)	1.02 ± 0.06	0.99 ± 0.05	0.879	1.14 ± 0.11	1.33 ± 0.34	0.357
Adiponectin (ng/ml)	10591 ± 670	11850 ± 710	0.798	5088 ± 575‡§	5928 ± 715‡§	0.586
Leptin (pg/mL)	2178 ± 823	2208 ± 708	0.796	1605 ± 211	1616 ± 368	0.976
Resistin (ng/ml)	4.34 ± 0.45	4.11 ± 0.36	0.596	4.25 ± 0.32	3.89 ± 0.52	0.692
ApoE genotype						
2-3	1	0	0.481	2	1	0.999
3-3	11	12	0.999	13	6	0.698
3-4	1	1	0.999	4	3	0.665
HFE mutation H63D heterozygotes (%)	16	8	0.159	18	20	0.892
Abdominal obesity (% subjects)	0	7	0.347	11	22	0.457
IGR (% subjects)	8	7	0.989	39*†	44*†	0.769
Hypertension (% subjects)	38	42	0.756	72†	55*†	0.478
Low HDL-C (%)	8	0	0.259	28*†	11†	0.964
High Tg (% subjects)	8	7	0.789	11	22*†	0.749
subjects with MS (%)	8	7	0.877	34*†	33*†	0.348
Steatosis (% hepatocyte)	–	–	–	41 ± 10	28 ± 9	0.001
Necroinflammatory grade	–	–	–	2.4 ± 1.1	1.3 ± 0.4	0.008
Fibrosis score	–	–	–	2.5 ± 1.2	1.1 ± 0.5	0.003

NOTE. Data are presented as mean ± SEM.

Abbreviations: BP, blood pressure; Br, bilirubin; ISI,insulin sensitivity index; total C, total cholesterol; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; WHR, waist-on-hip ratio; Tg, triglyceride; IGR, impaired glucose regulation.

*$P < 0.05$ versus controls GG.

†$P < 0.01$ versus controls GT.

‡$P < 0.001$ versus controls GG.

§$P < 0.001$ versus controls GT/TT.

tion containing 0.05% KBr/NaCl plus EDTA (pH 7.4); 3 ml of a similar solution at 1.020 g/ml; 3 ml of physiological saline at 1.006 g/ml. The sample was ultracentrifuged in a Beckman L8-70M centrifuge at 20°C in stages, allowing the separation of four VLDL fractions with decreasing Sf values: A > 400; B = 175-400; C = 100-175; D = 20-100. The first centrifugation (28,300 rpm for 43 minutes) isolated fraction A in a floating volume of 0.5 ml. The volume removed was replaced with physiological saline, and the sample was centrifuged at 40,000 rpm for 67 minutes to isolate fraction B. This procedure was then repeated at 40,000 rpm for 71 minutes and at 37,000 rpm for 18 hours to isolate fractions C and D, respectively. The automated methods mentioned were used to determine Chol and Tg on the 4 fractions.

ApoB48 and ApoB100 Analysis. TRLP ApoB48 and ApoB100 were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 3.9% gel. Nondelipidated samples were reduced in SDS buffer for 4 minutes at 96°C. Samples were applied to the gel

and run at 40 mA in 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. Gel was stained with Silver Stain (Bio-Rad). Because the chromogenicity of ApoB48 is similar to that of ApoB100, a protein standard was prepared from LDL isolated by sequential ultracentrifugation and used to quantify ApoB100 and ApoB48. The bands were quantified by densitometry using Gel Doc equipment (Bio-Rad). Density values were assigned to the ApoB100 bands of the standard LDL, and a standard curve was constructed. The values were recalculated by linear regression.

Markers of Lipid Peroxidation. LDL-conjugated dienes were determined during the fat load test, as follows. Capillary electrophoresis was performed as described by Stocks and Miller.[18] The cathode and anode electrolytes and the capillary run buffer were 40 mM methylglucamine-Tricine, pH 9.0. LDL samples were injected by low pressure for 4 seconds. Dimethylformamide was injected as an electroendosmotic flow marker for 1 second. A voltage of 24 kV was applied ramping over 0.8 minutes. Migration of LDL particles was monitored at 200 and 234 nm. The amount of conjugated dienes was obtained from the percentage of the height of LDL peak at 234 nm related to the height of LDL peak at 200 nm.

Statistical Analysis. Data were expressed as mean ± SEM. .Differences were considered statistically significant at $P < 0.05$. Differences between groups were analyzed by analysis of variance (ANOVA) when variables were normally distributed; otherwise the Mann-Whitney 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 area under the curve (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. Simple and multiple regression analyses were used to estimate linear relationship between different variables, after log transformation of skewed data. Logistic regression analysis was used to identify independent predictors for VLDL apoB48 and VLDL apoB100 subfraction responses. The covariates were waist, ISI, MTP polymorphism, adiponectin, and fasting insulin.

A logistic regression model was used to identify independent predictors for severe steatosis, necroinflammatory grade 3, or fibrosis stage 3. The covariates were age, waist, ISI, MTP polymorphism, adiponectin, IAUC-triglyceride/VLDL A apoB48/apoB100, IAUC-apoA1/LDL conjugated dienes.

Results

Subject Characteristics. The main features of NASH patients and controls grouped according to −493 MTP genotype are shown in Table 1.

The prevalence of −493 MTP G/G carriers was 48% in controls versus 66% in NASH ($P = 0.166$), heterozygous G/T carriers were 36% in controls versus 25% in NASH ($P = 0.558$), and homozygous TT carriers were 14% in controls versus 5% in NASH ($P = 0.665$), as described.[11] There was no significant difference in Apo E allelic frequency between the 4 groups. ISI, fasting insulin, and adiponectin were significantly lower in patients with NASH than in controls, but there was no difference between NASH GG and NASH GT/TT (Table 1).

Plasma high-density lipoprotein-C (HDL-C) was lower in NASH compared with controls, and homozygous GG had lower HDL-C levels than NASH GT/TT (Table 1). Plasma HDL2-C was similar in controls and NASH GT/TT, whereas it was lower in NASH GG compared with both NASH GT/TT and controls.

Four NASH GG and 2 NASH GT/TT had impaired glucose tolerance on oral glucose tolerance test, 2 NASH GG and 3 NASH GT/TT had had impaired fasting glycemia (fasting plasma glucose ≥ 110 mg/dl but < 126 mg/dl); the others had a normal glucose regulation (fasting plasma glucose < 110 mg/dl).

Adopting the ATP III criteria for definition of the metabolic syndrome,[19] 13 NASH GG and 5 NASH GT had hypertension (systolic/diastolic blood pressure ≥130/85 mm Hg), 2 NASH GG and 2 NASH GT/TT were hypertriglyceridemic (fasting plasma triglycerides ≥150 mg/dl), 5 NASH GG and 1 NASH GT/TT had low plasma HDL-C (HDL-C < 40 mg/dl in men and <50 mg/dl in women), 7 NASH GG and 4 NASH GT/TT had impaired glucose regulation (either impaired fasting glycemia, i.e., fasting plasma glucose ≥100 mg/dl but <126 mg/dl, or impaired glucose tolerance, i.e., plasma glucose ≥140 mg/dL at 2 hours on oral glucose tolerance test), and 2 NASH GG and 2 NASH GT/TT had abdominal obesity (waist circumference > 102 cm in men and >88 cm in women). Thirty-four percent of NASH GG and 33% of NASH GT/TT had the whole picture of the metabolic syndrome (at least 3 criteria met).

Histopathology. Fatty infiltration was mild (involving 5%-33% of hepatocytes) in 9 patients, moderate (33%-66% of hepatocytes) in 11 subjects, and severe (>66% of hepatocytes involved) in 9 patients. Necroinflammatory activity was grade 1 in 9 patients, grade 2 in 9, and grade 3 in 11.

Fibrosis was stage 0 in 5 patients, 1 in 7 patients, 2 in 8 patients, and 3 in 9 patients; cirrhotic changes were absent

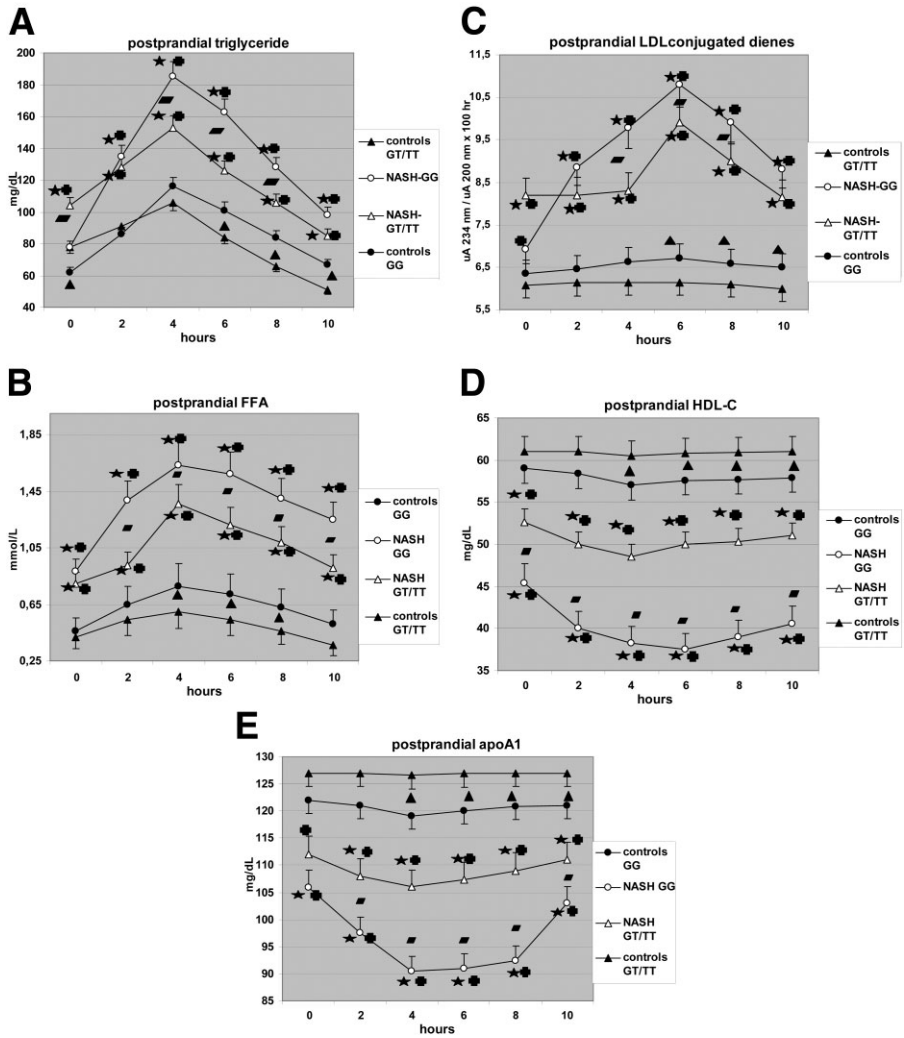

Fig. 1. Oral fat load test. Postprandial (A) plasma Tg, (B) FFA, (C) LDL conjugated dienes, (D) HDL-C, and (E) apoA1 responses in patients with NASH and controls. Data are presented as mean ± SEM. Star: $P < 0.05$ versus controls GG. Circle: $P < 0.05$ versus controls GT/TT. Rhombus: $P < 0.05$ NASH GG versus NASH-GT/TT. Triangle: $P < 0.05$ controls GG versus controls GT/TT.

(Table 1). Liver iron concentration was 15 ± 3 μmol/g dry weight, and hepatic iron index was 0.50 ± 0.04.

The severity of liver fatty infiltration, median necroinflammatory grade, and median fibrosis score were higher in homozygous GG carriers than in GT/TT genotype (Table 1).

Alimentary Record. Daily total energy and macronutrient intake of patients with NASH and controls were similar: total calories: 2,589 ± 132 versus 2,476 ± 145 kcal, $P = 0.875$; carbohydrate: 50 ± 2 versus 47 ± 2% kcal, $P = 0.476$; protein: 17 ± 3 versus 20 ± 2% kcal, $P = 0.568$; fat: 33 ± 2 versus 33 ± 1% kcal, $P = 0.879$. NASH group consumed more saturated fat and less polyunsaturated fat than controls, when expressed as both percentage of total calories and percent total fat intake: saturated fatty acids: 13.4 ± 0.8 versus 9.2 ± 0.5% tot kcal, $P = 0.000$; polyunsaturated fatty acids: 3.6 ± 0.3 versus 5.2 ± 0.4% tot kcal, $P = 0.000$.

NASH patients also had a significantly lower daily intake of antioxidant vitamin A (568 ± 176 vs. 1,078 ± 213 mg; $P = 0.008$), vitamin C (105 ± 12 vs. 156 ± 10 mg; $P = 0.005$) and E (5.7 ± 0.4 vs. 9.8 ± 0.4 mg, $P = 0.0002$), as previously reported.[17]

Daily alcohol intake was similar in the two groups: 8 ± 1 versus 9 ± 2 g; $P = 0.458$. No difference was seen in daily antioxidant intake or in any other macro/micronutrient intake between genotypes GG and GT/TT in NASH and controls (not shown).

Oral Fat Tolerance Test. Postprandial plasma total Tg and FFA responses were significantly higher in the NASH group than in controls, and homozygotes GG had significantly higher Tg and FFA responses than their counterpart GT/TT in both NASH and controls (Fig 1; Table 2).

Table 2. Oral Fat Load Parameters of Patients With NASH and Controls According to -493 G/T MTP Polymorphism

	Controls GG (n = 13)	Controls GT/TT (n = 14)	P Controls	NASH-GG (n = 19)	NASH-GT/TT (n = 10)	*P* NASH
Fasting Tg (mg/dl)*	62 ± 5	79 ± 6	0.041	78 ± 7*	104 ± 9*†	0.034
IAUC Tg (mg/dl × hr)*	179 ± 33	85 ± 31	0.038	552 ± 79‡§	277 ± 64*§	0.00001
Fasting FFA (mmol/l)	0.42 ± 0.08	0.46 ± 0.10	0.358	0.88 ± 0.32‡§	0.80 ± 0.20‡§	0.521
IAUC FFA (mmol/l × hr)	2.01 ± 0.31	1.06 ± 0.24	0.029	5.39 ± 2.10‡§	3.05 ± 1.09‡§	0.008
Fasting LDL C.D. (uA 234 nm/uA 200 nm × 100)	6.38 ± 1.41	6.07 ± 1.02	0.365	6.92 ± 1.43	8.18 ± 2.08*†	0.129
IAUC LDL C.D. (uA 234 nm/uA 200 nm × 100 × hour)	1.9 ± 0.5	0.4 ± 0.2	0.015	25.2 ± 5.1‡§	5.7 ± 3.9‡§	0.00002
Fasting HDL-C (mg/dl)	61 ± 1	59 ± 1	0.897	45 ± 1‡§	50 ± 2†§	0.004
IAUC HDL-C (mg/dl × hour)	−12 ± 2	−2 ± 2	0.002	−59 ± 8‡§	−26 ± 6‡§	0.005
Fasting ApoA1(mg/dl)	122 ± 4	127 ± 5	0.694	106 ± 4*†	112 ± 4†	0.354
IAUC ApoA1 (mg/dl)	−15 ± 2	−2 ± 2	0.002	−120 ± 18‡§	−36 ± 5‡§	0.006

NOTE. Data are presented as mean ± SEM.

Abbreviations: Tg, triglyceride; Glu, glucose; C, cholesterol; CD, conjugated dienes; adipo, adiponectin.

*$P < 0.05$ versus controls GG.

†$P < 0.01$ versus controls GT.

‡$P < 0.001$ versus controls GG.

§$P < 0.001$ versus controls GT/TT.

Similarly, LDL conjugated diene response was higher in patients with NASH than in controls, and homozygotes GG had a significantly higher postprandial IAUC LDL conjugated diene than GT/TT in both NASH and control groups (Fig. 1; Table 2).

Fasting and postprandial HDL-C was lower in NASH than in controls, and genotype GG had lower fasting and postprandial HDL-C levels than genotype GT/TT in both patients and controls. Fasting and postprandial apoAI levels were lower in NASH than in controls, and NASH GG had lower postprandial apoAI levels than NASH GT/TT.

The postprandial response of smaller intestinal and hepatic VLDL C and D was higher in GT/TT carriers than in homozygous GG in both patients and controls, but there was no difference in absolute values between NASH and controls (Table 3; Fig 2). LDL-C, insulin, and glucose levels did not change throughout the test (not shown).

Correlative Analysis. Main correlations between anthropometric, metabolic, and dietary parameters in patients with NASH are shown in Table 4. IAUC Tg correlated with adiponectin, ISI, fasting, and postprandial HDL-C, IAUC LDL conjugated dienes, and with postprandial intestinal ($r = 0.65$, $P = 0.0006$), and hepatic ($r = 0.43$, $P = 0.026$) VLDL A increase. On multiple regression analysis, only basal adiponectin ($\beta = -0.47$; $P = 0.009$) and IAUC VLDL A apo B48 ($\beta = 0.48$; $P = 0.008$) predicted IAUC-Tg. IAUC FFA correlated with postprandial intestinal and hepatic VLDL A increase, HDL-C, and with LDL conjugated diene increase On multiple regression analysis, only IAUC VLDL A apoB48 ($\beta = 0.47$; $P = 0.01$) predicted IAUC FFA. IAUC LDL conjugated dienes correlated with fasting adiponectin, postprandial IAUC Tg, and FFA, with postprandial intestinal and hepatic VLDL A and D apoB48 and apoB100 increase, and with dietary intake of saturated fatty acid and vitamin A (inversely). On multiple regression analysis, only IAUC VLDL A apoB48 ($\beta = 0.52$; $P = 0.002$) and vitamin A intake ($\beta = -0.40$; $P = 0.01$) predicted IAUC LDL conjugated dienes.

IAUC HDL-C correlated with IAUC Tg, IAUC apoA1, IAUC FFA, IAUC VLDL A apoB48, and IAUC VLDL A apoB100 on univariate analysis; on multiple regression analysis, only IAUC VLDL A apoB48 (0.42; $P = 0.009$) independently predicted IAUC HDL-C. Similarly, IACU apoA1 correlated with IAUC FFA($\beta = 0.40$; $P = 0.01$) and IAUC VLDL A apoB48 ($\beta = 0.51$; $P = 0.003$). On logistic regression analysis, MTP polymorphism (odds ratio [OR] = 3.5; confidence interval [CI] 1.8-5.8; $P = 0.0002$), and adiponectin (OR = 3.0; CI 1.8-4.0; $P = 0.0008$) predicted IAUC VLDL A apoB48, IAUC VLDL A apoB100 (for MTP polymorphism: OR = 2.8; CI 1.5-6.9; $P = 0.007$; for adiponectin: OR = 3.1; CI 1.9-4.9; $P = 0.0006$) and IAUC VLDL B apoB48 (for MTP polymorphism: OR = 2.0; CI 1.4-4.9; $P = 0.005$; for adiponectin: OR = 2.1; CI 1.5-3.9; $P = 0.004$).

MTP polymorphism predicted IAUC VLDL D apoB48 (OR = 2.8; CI 1.6-4.9; $P = 0.007$) and IAUC VLDL D apoB100 (OR = 2.2; CI 1.4-3.8; $P = 0.009$). Similarly, VLDL C apoB48 and apoB100 responses were independently predicted only by MTP polymorphism (not shown). Hepatic steatosis was independently predicted by MTP polymorphism (OR = 2.5; CI 1.8-7.9.4; $P = 0.018$), hypoadiponectinemia (OR = 4.1; CI 1.6-12.4; $P = 0.009$), and IAUC VLDL A apoB48 (OR = 2.4; CI 1.7-9.6; $P = 0.031$).

Table 3. Postprandial apoB Responses in VLDL Subfractions of Patients with NASH and Controls

	Controls GG (n = 13)	Controls GT/TT (n = 14)	P Controls	NASH-GG (n = 19)	NASH-GT/TT (n = 10)	P NASH
Fasting VLDL A ApoB48 (mg/dl)	0.33 ± 0.18	0.12 ± 0.10	0.010	1.33 ± 0.64‡§	0.62 ± 0.23*§	0.015
VLDL A IAUC ApoB48 (mg/dl × hr)	1.07 ± 1.04	0.47 ± 0.23	0.008	4.66 ± 1.21‡§	2.07 ± 1.00*†	0.0001
Fasting VLDL B ApoB48 (mg/dl)	0.40 ± 0.20	0.22 ± 0.13	0.010	0.85 ± 0.32‡§	0.61 ± 0.21*†	0.369
VLDL B IAUC ApoB48 (mg/dl × hr)	0.78 ± 0.17	0.32 ± 0.13	0.009	3.48 ± 1.02‡§	1.43 ± 0.21*†	0.0001
Fasting VLDL C ApoB48 (mg/dl)	1.36 ± 0.41	1.42 ± 0.38	0.841	0.48 ± 0.21‡§	0.44 ± 0.27‡§	0.754
VLDL C IAUC ApoB48 (mg/dl × hr)	2.79 ± 1.38	5.51 ± 1.04	0.001	2.91 ± 1.07‡§	5.69 ± 1.43‡	0.0001
Fasting VLDL D ApoB48 (mg/dl)	1.89 ± 0.35	2.49 ± 0.35	0.007	0.74 ± 0.38§	1.48 ± 0.47†	0.0001
VLDL D IAUC ApoB48 (mg/dl × hr)	5.22 ± 1.53	9.41 ± 2.02	0.006	4.63 ± 1.07‡§	8.66 ± 2.81*	0.0001
Fasting VLDL A ApoB100 (mg/dl)	1.06 ± 0.08	0.17 ± 0.08	0.0001	4.38 ± 0.85‡§	2.39 ± 1.21‡§	0.0001
VLDL A IAUC ApoB100 (mg/dl × hr)	2.68 ± 1.25	1.26 ± 0.84	0.009	13.73 ± 2.65‡§	6.58 ± 1.53‡§	0.001
Fasting VLDL B ApoB100 (mg/dl)	1.40 ± 0.36	0.71 ± 0.12	0.010	2.33 ± 1.19*†	1.21 ± 0.38†	0.036
VLDL B IAUC ApoB100 (mg/dl × hr)	5.21 ± 1.17	2.42 ± 0.86	0.0007	8.27 ± 2.93‡	5.27 ± 1.16	0.002
Fasting VLDL C ApoB100 (mg/dl)	4.87 ± 1.49	5.25 ± 1.51	0.219	0.69 ± 0.25‡§	3.33 ± 1.11‡§	0.0003
VLDL C IAUC ApoB100 (mg/dl x hr)	3.96 ± 1.14	6.80 ± 2.01	0.007	4.01 ± 1.79†	6.54 ± 1.85*	0.003
Fasting VLDL D ApoB100 (mg/dl)	6.70 ± 1.26	9.21 ± 1.57	0.003	1.47 ± 0.85‡§	5.07 ± 1.19§	0.0002
VLDL D IAUC ApoB100 (mg/dl × hr)†	6.62 ± 2.76	17.56 ± 3.76	0.0004	5.58 ± 2.18§	18.81 ± 4.19‡	0.0002

NOTE. Data are given as mean ± SEM.
*$P < 0.01$ versus controls GG.
†$P < 0.01$ versus controls GT.
‡$P < 0.001$ versus controls GG.
§$P < 0.001$ versus controls GT/TT.

Necroinflammatory grade 3 was predicted by fasting adiponectin (OR = 4.2; CI 1.5-16.0; $P = 0.008$) and IAUC-LDL coniugated dienes (OR = 3.3; CI 1.4-9.7; $P = 0.016$). Stage 3 fibrosis was predicted by fasting adiponectin (OR = 5.0; CI 2.0-11.0; $P = 0.006$), IAUC apoA1 (OR = 2.1; CI 1.5-4.0; $P = 0.015$) and IAUC-LDL conjugated dienes (OR = 2.8; CI 1.0-8.5; $P = 0.030$).

Discussion

The main findings of our study are the following: (1) despite comparable adiposity, insulin resistance and adipokine profile, and lower fasting Tg levels, homozygous MTP −493 GG carriers of both NASH and controls had a higher postprandial lipemia and LDL lipid peroxidation and a more pronounced postprandial HDL-C and apoA1 fall than their counterpart genotype GT/TT; the magnitude of postprandial lipemia and lipid peroxidation predicted the severity of liver histology in NASH.

(2) The higher postprandial lipemia in GG homozygosity was accounted for by a shift of circulating TRLPs toward the larger, more Tg-rich subfractions. The large TRLP subfractions response was related to MTP polymorphism and to adiponectin, whereas smaller TRLP responses are solely related to MTP polymorphism.

The association of −493 G/T genotype with liver histology in our patients confirms previous reports[10]: −493 MTP GG homozygosity is more prevalent in NAFLD than in the general population and has been considered a risk factor for NAFLD; however, its role in the pathogenesis of fatty liver is unclear.[1,10] Although this study is not powered enough to detect different prevalences in MTP genotypes between groups, the absence of significant fatty liver in controls suggests MTP GG polymorphism may not be per se sufficient

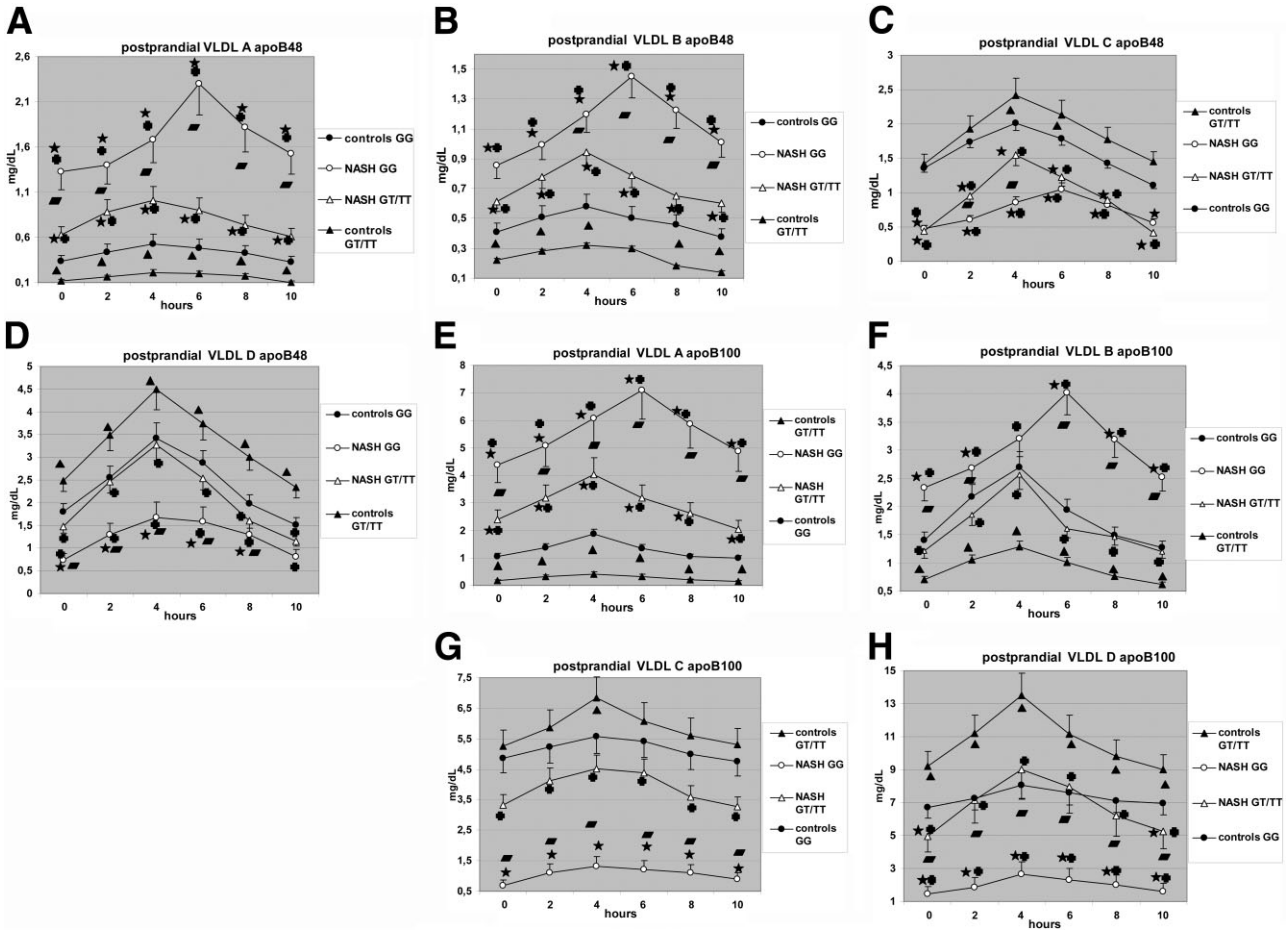

Fig. 2. Oral fat load test. Postprandial VLDL apoB48 (A-D) and apoB100 (E-H) subfraction responses in patients with NASH and controls. Data are presented as mean ± SEM. Star: $P < 0.05$ versus controls GG. Circle: $P < 0.05$ versus controls GT/TT. Rhombus: $P < 0.05$ NASH GG versus NASH-GT/TT. Triangle: $P < 0.05$ controls GG versus controls GT/TT.

to cause NASH; rather, it is a modifier gene in NASH, significantly impacting liver disease only when other "environmental" factors (insulin resistance, hypoadiponectinemia, reduced antioxidant intake) superimpose.

MTP polymorphism may affect NASH both directly, by modulating MTP activity and consequent assembly and secretion of hepatic VLDL,[10] and indirectly, in a more complex way, by affecting postprandial lipemia and oxidative stress.[9] Furthermore, the ability of MTP to bind stored Tg and facilitate their lipolysis and oxidation has been recently hypothesized.[20]

The liver is an important scavenger of LDL and intestinally derived remnants through the LDL- receptor and the liver-related receptor protein; furthermore, hepatic FFA uptake is driven exclusively by their plasma concentration and contributes substantially to liver Tg pool in NAFLD and diabetes.[7,8] Our data link the postprandial increase in TRLP and LDL dienes to the severity of liver histology in NASH, suggesting that postprandial lipemia may modulate oxidative liver injury in these subjects. Consistently, oxidized LDL can bind to scavenger the receptors CD36 of Kupffer and stellate cells, triggering inflammatory cascade and extracellular matrix deposition[21]; the amount of oxidized LDL and the degree of CD36 expression on activated stellate cells paralleled the expression of the profibrotic cytokine transforming growth factor beta and the severity of fibrosis in NASH.[22,23] Finally, an increased intracellular oxidative stress experimentally impaired VLDL secretion and led to Tg accumulation in hepatocytes, an effect totally reversed by antioxidant treatment.[24]

The postprandial phase was suggested to be a major atherogenic risk factor in diabetes.[6] During this phase TRLPs are hydrolyzed to remnants by lipases, LDL are enriched with peroxidation-prone triglyceride, and HDLs are depleted of cholesterol via cholesterol-ester transfer protein. The proatherogenic effects of oxidized LDL and TRLP-remnants on vascular endothelium and macrophages, as well as the protective role of HDL-C, are well documented[6]: among metabolic syndrome components,

Table 4. Pearson Correlation Coefficients Between Different Variables in Patients with NASH

	HDL-C	HDL2-C	IAUC apoA1	ISI	Adiponectin	IAUC Tg	IAUC FFA	IAUC LDL C.D.	IAUC VLDL A apoB48 IAUC-Tg	IAUC VLDL D apoB48	IAUC VLDL A ApoB100	IAUC VLDL D ApoB100	IAUC HDL-C	Vit A	SFA % E
HDL-C	-	**0.68‡**	0.21	**0.50†**	**0.47***	**−0.42***	**−0.50***	0.12	**−0.45***	0.22	**−0.48***	−0.22	0.11	0.35	−0.20
HDL2-C	0.68‡	-	0.35	0.60‡	**0.48***	**−0.43***	**−0.45***	0.31	**−0.43***	0.18	**−0.53†**	0.31	0.38	0.12	−0.10
IAUC apoA1	0.21	0.35	-	0.32	**0.45***	−0.40	**−0.61‡**	**0.50†**	**−0.63‡**	**0.42***	0.21	**−0.60‡**	**0.44***	0.28	0.31
ISI	0.50†	**0.60‡**	0.32	-	**0.59‡**	**−0.55‡**	−0.39	−0.32	−0.38	**0.45***	−0.35	0.41	−0.24	0.14	−0.38
Adipo.	0.47*	**0.48***	**0.45***	**0.59‡**	-	**−0.74‡**	−0.36	**−0.51‡**	**−0.65‡**	**−0.42***	**−0.60‡**	**−0.48***	−0.26	0.13	0.35
IAUC-Tg	−0.42*	**−0.43***	−0.40	**−0.55‡**	**−0.74‡**	-	0.39	**0.72‡**	**0.65‡**	0.32	**0.43‡**	0.36	**0.55‡**	−0.14	0.31
IAUC FFA	−0.50†	−0.45	**−0.61‡**	−0.39	−0.36	0.39	-	**0.55‡**	**0.73‡**	0.32	**0.43†**	0.31	**−0.45***	−0.26	0.23
IAUC LDLC.D.	0.12	0.31	**0.50†**	−0.32	**−0.51†**	**0.72‡**	**−0.55†**	-	**0.58‡**	**0.61‡**	**0.42**	−0.50†	−0.33	**−0.50†**	**0.64‡**
IAUC VLDL A apoB48	−0.45*	**−0.43***	**−0.63‡**	−0.38	**−0.65‡**	**0.65‡**	**0.73‡**	**0.58‡**	-	0.11	**0.61‡**	0.22	**−0.68‡**	−0.26	−0.39
IAUC VLDL D ApoB48	0.22	0.18	**0.42***	**0.45***	**−0.42***	0.32	0.32	**−0.61‡**	0.11	-	0.15	0.22	−0.23	**0.60‡**	0.18
IAUC VLDL A ApoB100	−0.48*	**−0.53†**	0.21	−0.35	**−0.60‡**	**0.43***	**0.43***	0.42	**0.61‡**	0.15	-	0.33	**−0.42†**	−0.32	**−0.50†**
IAUC VLDL D ApoB100	0.22	0.31	**0.60‡**	**0.41**	−0.48*	0.34	0.31	**−0.50†**	**0.22**	0.22	0.33	-	−0.31	0.25	0.28
IAUC HDL-C	0.11	0.38	**0.44***	−0.24	−0.26	**−0.55‡**	**0.45***	−0.33	**−0.68‡**	−0.23	**0.42***	−0.31	-	−0.20	0.15
vit A	0.35	0.12	0.28	0.14	0.13	−0.14	−0.26	**−0.50†**	−0.26	**0.60‡**	−0.32	0.25	−0.20	-	0.24
SFA %E	−0.20	−0.10	0.31	−0.38	0.35	0.31	0.23	**0.64‡**	−0.39	0.18	**−0.50†**	0.28	0.15	0.24	-

NOTE. Statistically significant correlations are written in bold characters.

Abbreviations: Tg, triglyceride; Glu, glucose; C, cholesterol; C.D., conjugated dienes; adipo, adiponectin.

*$P < 0.05$.

†$P < 0.01$.

‡$P < 0.001$.

in particular, low HDL-C predicted the highest cardiovascular risk.[25] In our subjects, GG homozygosity displayed a higher postprandial Tg, FFA, and LDL conjugated diene increase and a more pronounced decrease in circulating HDL-C and apoA1 than GT/TT genotype counterpart despite comparable adiposity, insulin resistance, adipokines, and lower fasting Tg levels. In our subjects, excess of postprandial intestinal VLDL A particles likely enhanced LDL lipid peroxidation and altered the lipid composition of HDL particles, increasing apoA1 catabolism and eventually lowering HDL-C levels,[26] as suggested by the independent correlation of postprandial VLDL A apo B48 with LDL conjugated dienes, HDL-C, and apoA1 responses. The higher postprandial lipemia, LDL lipid peroxidation, and the deeper postprandial HDL-C decrease of NASH GG compared with NASH GT/TT may confer on these patients an increased cardiovascular risk, as recently reported in overweight subjects,[27] independently of classical risk factors, insulin resistance, and metabolic syndrome,[3-5] and provide a pathogenetic mechanism for the inverse association between apoA1 levels and the severity of liver histology in NASH.[28]

TRLP subfraction analysis disclosed that the higher postprandial lipemia in homozygous GG versus GT/TT genotypes is accounted for by the accumulation of larger, more Tg-rich subfractions in both patients and controls. The effect of this polymorphism on TRLP metabolism in NAFLD has not been previously evaluated.

An elevated MTP activity has been linked to a higher number of plasma smaller TRLP in diabetes in fasting conditions.[29-31]Others found a higher number of fasting circulating particles in both large and small TRLP subfractions in GG versus TT genotype in healthy subjects.[11] Another study in diabetic patients found a prevalence of larger TRLP in MTP GG versus TT genotype postprandially.[32] Accordingly, in our subjects the postprandial lipemic response of GG carriers consists of larger TRLPs, whereas smaller subfractions C and D account for the lipemic response of GT/TT carriers in both patients and controls.

Whether the accumulation of large TRLPs in GG genotype is due to enhanced secretion or delayed clearance cannot be elucidated without kinetic studies. Because ISI, insulin, and adiponectin were similar in GG and GT/TT genotypes and plasma insulin and glucose did not change throughout the test (not shown) in both patients and controls, differences in the inhibitory effect of insulin on VLDL secretion are unlikely to contribute to the different lipemic response of MTP GT genotypes.

Notably, obese MTP GG carriers showed a 35% higher VLDL apoB100 secretion rate than the other genotypes.[33] Furthermore, hepatic Tg availability seems a major determinant of large VLDL secretion rate in insulin resistance.[34,35] In the presence of liver fat accumulation by different causes (defective FFA β-oxidation, defective VLDL secretion, increased FFA and Tg uptake), increased Tg availability plus a reduced MTP activity may

prolong the residence time of nascent TRLP in the cell, allowing Tg enrichment of TRLPs; larger TRLPs finally may accumulate in the circulation because of delayed clearance; a higher MTP activity expedites secretion of a larger number of small TRLPs, which are cleared more quickly from the circulation.[36] The postprandial accumulation of large TRLP is magnified in a setting of higher substrate (i.e., Tg, FFA) availability and defective TRLP clearance such as the insulin-resistant state. The association of hypoadiponectinemia with postprandial lipemia and large TRLP responses is consistent with the ability of this adipokine to enhance LPL-mediated TRLP catabolism and FFA oxidation.[1,37]

These findings highlight the key role of Tg-rich lipoprotein subfractions, beyond simple fasting lipid levels, in determining postprandial metabolism and suggest the combined impact of MTP activity and adipokines on lipoprotein metabolism may be much more complex than the picture that emerged from studies in fasting conditions: despite lower fasting Tg levels, in fact, homozygous GG carriers had a higher postprandial lipemia than the GT/TT counterpart.

In conclusion, our study shows −493 GT MTP polymorphism significantly impacts liver disease and postprandial lipid metabolism in NASH: for similar degrees of adiposity, insulin resistance, adipokine profile, and similar dietary habits, patients with GG homozygosity had more severe liver disease and more atherogenic postprandial lipoprotein profiles than the other genotypes. Postprandial metabolism of large chylomicrons is a major determinant of postprandial Tg, FFA, LDL, and HDL responses and may have an important role in peroxidative liver injury and in postprandial proatherogenic changes in NASH.

If the increased cardiovascular risk of homozygous GG carriers with NAFLD is prospectively confirmed by larger studies, assessing this MTP polymorphism may help select those subjects amenable to more aggressive, pharmacological intervention targeting postprandial lipid metabolism (i.e., statins, PPAR-alpha agonists). Furthermore, our data suggest a reduced hepatic MTP function, as obtained by MTP inhibitors, a novel class of hypolipemic drugs currently under investigation, may be steatogenic and convey highly atherogenic postprandial lipoprotein changes.

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

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