Transcription Factor 7–Like 2 Polymorphism Modulates Glucose and Lipid Homeostasis, Adipokine Profile, and Hepatocyte Apoptosis in NASH

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Genetic factors underlying the association of NAFLD with diabetes and atherosclerosis are unknown. Recent human studies suggest transcription factor 7–like 2 (TCF7L2) polymorphism predisposes to diabetes through modulation of β-cell function and modulates lipid levels in familial dyslipidemia. Emerging experimental evidence connects TCF7L2 to adipocyte metabolism and lipid homeostasis, as well. We tested if TCF7L2 polymorphism is a risk factor for nonalcoholic fatty liver disease (NAFLD) and if it modulates liver injury, glucose homeostasis, lipoprotein, and adipokine profiles in NASH. TCF7L2 genotype and dietary habits of 78 nondiabetic normolipidemic NAFLD subjects and 156 age-, body mass index–, sex-matched healthy controls were assessed. In 39 biopsy-proven nonalcoholic steatohepatitis (NASH) and matched controls TCF7L2 polymorphism was correlated to liver histology and oral glucose tolerance test–derived parameters of glucose homeostasis. Patients with NASH and controls consumed a high-fat meal and TCF7L2 genotype was correlated to postprandial circulating lipoproteins, adipokines, and cytokeratin-18 fragments. The TCF7L2 CT/TT genotype was more frequent in NAFLD and predicted the presence and severity of liver disease, of β-cell dysfunction, of reduced incretin effect and hepatic insulin resistance in NASH; it also modulated postprandial hepatocyte apoptosis, lipoproteins, and adipokine profiles in both groups. *Conclusion:* TCF7L2 polymorphism predisposes to NAFLD and significantly impacts liver injury, glucose homeostasis, and postprandial lipoprotein and adipokine responses to fat ingestion. This polymorphism also modulates a fat-induced increase in circulating markers of hepatocyte apoptosis in NASH. Targeting postprandial lipemia, at least in at-risk TCF7L2 genotypes, may improve liver disease and glucose dysmetabolism in these patients. (HEPATOLOGY 2009;49:426-435.)

Nonalcoholic fatty liver disease (NAFLD) is the main cause of cryptogenic cirrhosis and predisposes to diabetes and cardiovascular disease, independently of insulin resistance, metabolic syndrome, and traditional risk factors.[1,2] The pathogenesis of NAFLD is unclear, and varied therapeutic approaches (i.e., weight loss, insulin sensitizers, incretin mimetics, hypolipemizers, antioxidants) targeting the most prominent accompanying metabolic disorder are under evaluation.[3,4]

A genetic background is indisputably present in NAFLD; the identification of genes predisposing to NAFLD and to the associated cardio-metabolic risk would allow earlier and more individualized treatments. Several genes modulating lipid and glucose metabolism have been experimentally linked to NAFLD and to atherosclerosis and diabetes in the general population.[5-7]

Rs7903146C/T polymorphism in transcription factor 7–like 2 (TCF7L2) has been associated to diabetes in the general population through β-cell dysfunction and impaired incretin action, with allele T being at risk.[8] TCF7L2 polymorphism modulates fasting lipid levels in familial hyperlipidemia and is differentially expressed in adipocytes of diabetic and dyslipidemic subjects, suggesting this polymorphism may regulate adipokine secretion

Abbreviations: AUC, area under the curve; BMI, body mass index; CI, confidence interval; CK-18, cytokeratin-18; IAUC, incremental area under the curve; LDL, low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OGTT, oral glucose tolerance test; OR, odds ratio; TCF7L2, transcription factor 7–like 2; Tg, triglyceride; VLDL, very low-density lipoprotein.

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and lipid metabolism as well.[9,10] Whether TCF7L2 predisposes to NAFLD and modulates lipoprotein metabolism and adipokine profile in these subjects is currently unknown.

We assessed the impact of TCF7L2 polymorphism and dietary habits on the risk and severity of liver disease, on glucose homeostasis, and on adipokine responses to a fat meal in patients with NAFLD. Next, we tested if this polymorphism modulates fat-induced hepatocyte apoptosis in these patients.

Obese and diabetic subjects were excluded, because we aimed at identifying early mechanisms predisposing to cardio-metabolic disease, and different adipokines may intervene as diabetes, dyslipidemia, and obesity appear. Postprandial glucose and lipoprotein metabolism were assessed, because postprandial dysmetabolism is an established cardiovascular risk factor; furthermore, exaggerated postprandial lipemia contributes substantially to liver triglyceride (Tg) accumulation in NAFLD and is not readily accessible in routine clinical practice.[11,12]

Patients and Methods

Patient Selection. Based on available data, we considered a type I error of 0.05 and a type II error of 0.20: at least 70 subjects per arm were needed to detect a significant difference in the prevalence of TCF7L2 polymorphism between NAFLD and controls, and at least 33 subjects per arm were needed to detect differences in parameters of glucose and lipoprotein metabolism between the two groups.[8,9,11]

Among 214 patients referred by family physicians to our Hepato-Metabolic Clinic for chronic liver enzyme elevations during 2006–2008, 78 subjects with NAFLD were identified. NAFLD was defined according to the following criteria: persistently (>6 months) elevated liver enzymes; ultrasonographic bright liver without any other liver or biliary tract disease; negative viral markers; and a history of alcohol consumption <20 g/day in men and <10 g/day in women as assessed by a detailed interview extended to family members and by a validated questionnaire filled in daily for 1 week by the patients.

Other exclusion criteria were obesity (body mass index [BMI] $\geq$30 kg/m^2), diabetes (fasting plasma glucose $\geq$126 mg/dL or plasma glucose $\geq$200 mg/dL at +2 hours on oral glucose tolerance test [OGTT]), or clinically overt atherosclerosis; exposure to occupational hepatotoxins or drugs known to be steatogenic or hepatotoxic or that affect lipid/glucose metabolism; positive autoimmune or celiac disease markers; abnormal copper metabolism, serum α_1-antitripsin, or thyroid function; and overt dyslipidemia (fasting serum cholesterol $\geq$200 mg/dL or plasma triglyceride $\geq$200 mg/dL). Mutations in the hemochromatosis genes HFE and TRF2 were detected in patients and controls using multiplex amplification reaction (Nuclear Laser Medicine, Milan, Italy). To select which patients should be proposed for liver biopsy, the NAFLD fibrosis score was used. The NAFLD fibrosis score has been recently validated as a method of selecting for biopsy those patients who might have progressive liver disease, avoiding biopsy of pure, nonprogressive fatty liver.[13] The mean $\pm$ standard error fibrosis score of the NAFLD group was 0.67 $\pm$ 0.22. To ensure biopsying subjects with the progressive form of NAFLD (i.e., NASH), liver biopsy was proposed for patients with a high (>0.676) or indeterminate (−1.455-0.676) NAFLD fibrosis score. Thirty-nine patients met these criteria, and 34 of these patients agreed to undergo liver biopsy. All 34 patients received a histological diagnosis of NASH.[14] Liver iron concentration and hepatic iron index were assessed from 2 mg dry weight tissue via atomic absorption spectroscopy.

Genetic Analyses. Patients and controls were genotyped for TCF7L2 rs7903146 C/T and apolipoprotein E polymorphisms. Genotyping for the TCF7L2 single nucleotide polymorphism rs7903146 incorporated the real-time allele discrimination method using the TaqMan Allelic Discrimination Assay (Applied Biosystems, Foster City, CA). The TaqMan genotyping reaction was run using a 7300HT real-time polymerase chain reaction (Applied Biosystems). The apolipoprotein E genotype was determined via polymerase chain reaction amplification of genomic DNA using specific oligonucleotide primers.

Dietary Intake. Subjects completed a daily dietary record for 1 week according to the EPIC protocol. Patients and controls were instructed to fill in a 7-day, extensively validated and reproducible diet record during an individual training session with a nutritionist. A list of foods was designed, and different portion sizes were specified for each item according to the EPIC study.[15] The recorded period included a complete week. The diet record was analyzed using the WinFood database (Medimatica, Teramo, Italy) according to the table of food consumption of the Italian National Institute of Nutrition and Food Composition Database for Epidemiological Study in Italy.[16,17]

Anthropometry. Percent body fat was estimated by the BIA method (TBF-202, Tanita, Tokyo, Japan), closely correlating with dual X-ray absorption.[18]

Cytokines. Serum tumor necrosis factor α, leptin, resistin, and adiponectin were measured by immunoenzymatic methods (see the Supplementary Material and Methods online).

Controls. Out of 1,100 healthy subjects enrolled in a population-based cohort study, 156 nonobese nondiabetic normolipidemic healthy subjects matched for age, sex, BMI, and waist were randomly identified. To further rule out subclinical liver disease in controls, in addition to a negligible alcohol intake (<20 g/day in men and <10 g/day in women) and normal abdomen ultrasound, upper healty ALT limit was lowered to <30 U/L (males) and <20 U/L (females).[19,20]

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

OGTT-Derived Indexes of Glucose Homeostasis. After completion of the alimentary record, patients selected for liver biopsy and 39 matched controls underwent a standard 75-g OGTT, and indexes of glucose homeostasis were calculated.

Areas under the curves (AUCs) 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-minute AUC of C-peptide divided by the 30-minute increase in circulating glucose. Whole body insulin sensitivity was estimated from a model of glucose clearance, which provides OGIS, an index of whole body insulin sensitivity that has been validated against clamp in nondiabetic subjects.[21] The hepatic insulin resistance index was calculated from OGTT as proposed and validated against clamp in nondiabetic subjects.[22] The hepatic insulin extraction (He), as a percentage of secreted hormone, was estimated using the formula [1 − (AUC insulin/AUC C-pep)].

Two OGTT-derived indexes of β-cell function, the insulinogenic index, computed as the suprabasal serum insulin increment divided by the corresponding plasma glucose increment in the first 30 minutes (ΔI30/ΔG30), and the CP-genic index (computed as ΔC-pep30/ΔG30), previously validated against measures of β-cell function derived from frequently sampled intravenous glucose tolerance test, were calculated.[23-25]

β-Cell ability to adapt insulin secretion to changes in insulin sensitivity was assessed by two indexes, the disposition index and the adaptation index, calculated by multiplying insulin sensitivity indexes (OGIS) by insulinogenic index and CP-genic index, respectively. These indexes relate β-cell insulin secretion to insulin resistance and represent integrated parameters of β-cell function, validated against frequently sampled intravenous glucose tolerance test MINIMAL MODEL parameters in NAFLD by us (unpublished data) and in nondiabetic subjects by other groups[26,27]; they also accurately predict future type 2 diabetes in the general population.[28]

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

Oral Fat Load. Within 2 weeks of the glucose tolerance tests, patients with biopsy-proven NASH and matched controls underwent an oral high-fat load test as described.[29] Samples were drawn at 2 hours intervals for 10 hours. Plasma total cholesterol, Tg, and free fatty acids were measured using automated enzymatic methods. Plasma resistin, adiponectin, insulin, and glucose were measured at each time period during the oral fat load.

Tg-Rich Lipoprotein Subfractionation and Low-Density Lipoprotein Lipoperoxidation Measurement. Tg-rich lipoprotein was isolated via preparative ultracentrifugation and subfractionated. Low-density lipoprotein (LDL)-conjugated dienes were determined as markers of lipid peroxidation (see the Supplementary Material and Methods online).

Hepatocyte Apoptosis. To assess whether fat ingestion promotes acute hepatocyte apoptosis, plasma hepatic caspase-3–generated cytokeratin-18 (CK-18) fragments were measured by M30-Apoptosense ELISA kit. The M30-Apoptosense ELISA kit, a one-step in vitro immunoassay for the quantitative determination of the apoptosis-associated CK-18Asp396 neo-epitope in serum (PEVIVA AB, Bromma, Sweden), has a sensitivity of 25 U/L in a 25-μL sample size and a range of 75-1,000 U/L. The intra- and interassay coefficients of variation (CVs) are less than 8%.

CK-18 is a major cytoplasmatic intermediate filament protein in hepatocytes and is cleaved by caspases at the C-terminal domain, resulting in exposure of an epitope called M30-antigen. Due to their specificity to hepatocytes, plasma CK-18 fragments levels have been validated as an accurate marker of necroinflammation in NAFLD and correlate tightly and specifically with hepatocyte apoptosis and hepatic necroinflammation.[30,31]

Statistical Analysis. Differences between groups were analyzed by analysis of variance, followed by Bonferroni correction, for multiple comparisons of normal variables. Otherwise, the Kruskal-Wallis test, followed by a post hoc Dunn test, was used for nonparametric variables. Normality was evaluated with a Shapiro-Wilk test. The chi-square test was used to compare categorical variables.

The AUC and incremental AUC (IAUC) of different lipid parameters, adiponectin, and resistin levels during the oral fat load and of glucose, insulin, and C-peptide during the glucose tolerance tests were computed by the

Table 1. Baseline Characteristics of Patients with NAFLD Grouped According to rs7903146C/T TCF7L2 Polymorphism

	Control			NAFLD		
	TCF CC (n = 79)	TCF CT/TT (n = 77)	*P* Value	TCF7L2 CC (n = 19)	TCF7L2 TT/CT (n = 59)	*P* Value
Age (years)	40 ± 2	41 ± 2	0.814	38 ± 2	39 ± 2	0.875
Sex (% males)	69	71	0.911	77	73	0.647
Smokers (%)	34	36	0.746	40	31	0.523
Body mass index (kg/m^2)	25.5 ± 0.8	25.2 ± 0.9	0.734	25.2 ± 0.6	24.9 ± 0.5	0.643
% Body fat	22 ± 3	21 ± 3	0.645	23 ± 2	22 ± 2	0.435
Waist circumference (cm)	89 ± 2	88 ± 3	0.423	91 ± 2	90 ± 2	0.326
Waist-to-hip ratio	0.92 ± 0.02	0.91 ± 0.03	0.435	0.92 ± 0.03	0.93 ± 0.02	0.401
Systolic blood pressure (mm Hg)	127 ± 2	124 ± 2	0.508	125 ± 2	128 ± 3	0.349
Diastolic blood pressure (mm Hg)	77 ± 2	78 ± 2	0.675	87 ± 2‡,§	88 ± 2‡,§	0.713
Tg (mg/dL)	88 ± 6	91 ± 6	0.541	88 ± 9	94 ± 8	0.286
Free fatty acids (mmol/L)	0.63 ± 0.11	0.65 ± 0.13	0.859	0.91 ± 0.25*,†	0.92 ± 0.28*,†	0.973
Total cholesterol (mg/dL)	179 ± 9	174 ± 8	0.121	179 ± 8	178 ± 8	0.873
HDL cholesterol (mg/dL)	62 ± 1	59 ± 1	0.045	52 ± 1‡,§	46 ± 1‡,§	0.031
LDL cholesterol (mg/dL)	105 ± 8	107 ± 9	0.393	110 ± 7	112 ± 8	0.411
Serum creatinine (mg/dL)	1.00 ± 0.09	1.00 ± 0.09	0.867	0.8 ± 0.2	0.9 ± 0.1	0.887
Glucose (mg/dL)	91 ± 3	93 ± 3	0.356	94 ± 2	95 ± 2	0.679
Insulin (μU/mL)	5.5 ± 1.8	6.6 ± 1.9	0.291	10.3 ± 2.4‡,§	12.6 ± 2.0‡,§	0.238
AST (U/L)	15 ± 2	14 ± 2	0.854	32 ± 4	34 ± 3	0.213
ALT (U/L)	17 ± 2	18 ± 2	0.645	62 ± 7‡,§	81 ± 6‡,§	0.112
GGT (U/L)	26 ± 4	29 ± 4	0.341	46 ± 5*,†	78 ± 6‡,§	0.092
TNF-α (pg/mL)	1.19 ± 0.12	1.26 ± 0.10	0.232	1.29 ± 0.11	1.24 ± 0.09	0.216
Adiponectin (ng/mL)	10,259 ± 813	11,047 ± 729	0.634	5,928 ± 611‡,§	5,058 ± 506‡,§	0.885
Leptin (pg/mL)	2,135 ± 791	2,079 ± 813	0.781	1,618 ± 219	1,605 ± 183	0.901
Resistin (ng/mL)	3.44 ± 0.29	3.51 ± 0.20	0.234	3.82 ± 0.36	3.64 ± 0.33	0.535
Apolipoprotein E genotype (%)						
2–3	14	12	0.314	13	12	0.811
3–3	68	67	0.756	69	67	0.540
3–4	18	21	0.541	18	21	0.417
Metabolic syndrome (% subjects)	6	7	0.534	42	46	0.518
Steatosis (% hepatitis)	–	–	–	21 ± 3	45 ± 6	0.005
Necroinflammatory grade	–	–	–	1.2 ± 0.1	2.5 ± 0.2	0.0006
Fibrosis stage	–	–	–	1.0 ± 0.1	2.4 ± 0.2	0.0008

Data are expressed as the mean ± standard error of the mean.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TNF-α, tumor necrosis factor α.

*$P < 0.05$ versus control TCF7L2 CT/TT.

†$P < 0.05$ versus control TCF7L2 CC.

‡$P < 0.001$ versus control TCF7L2 CT/TT.

§$P < 0.001$ versus control TCF7L2 CC.

trapezoid method. Data from the oral fat load were compared via analysis of variance and Scheffè post hoc test after log normalization of skewed variables. Data are expressed as the mean ± standard error of the mean. Differences were considered statistically significant at $P < 0.05$.

TCF7L2 polymorphism was modeled as an additive effect—that is, quantitative predictor variables reflecting the number of risk alleles (0, 1, or 2)—as defined previously.[32,33] Analysis of genetic polymorphisms, together with dietary, anthropometric, and metabolic parameters, was made using nonparametric Spearman rank correlation test. Discrete variables were divided into classes for analysis. When a relation was found on univariate analysis, multiple regression analyses were used to estimate the relationship between different variables after log transformation of skewed data.

A logistic regression model was used to identify independent predictors for severe (>66% hepatocytes) steatosis, necroinflammatory grade 3, or fibrosis stage 3. The covariates were age, BMI, waist, OGIS, TCF7L2 polymorphism, fasting and postprandial adiponectin/resistin/CK-18, and IAUC-triglyceride/very low-density lipoprotein (VLDL) 1 apoB48/apoB100/LDL conjugated dienes.

Results

Subjects Characteristics. The main features of patients with NAFLD grouped according to rs7903146 C/T genotype are reported in Table 1.

The prevalence of TCF C/C carriers was 19% in NAFLD versus 51% in controls ($P = 0.0001$), heterozygous C/T carriers were 69% in NAFLD versus 45% in

Table 2. OGTT-Derived Indexes of Glucose Homeostasis of Patients with NASH According to TCF7L2 rs7903146C/T Polymorphism

	Control			NASH		
	TCF CC (n = 20)	TCF CT/TT (n = 19)	*P* Value	TCF7L2 CC (n = 13)	TCF7L2 TT/CT (n = 26)	*P* Value
OGIS (mL · min^{-1} · m^{-2})	458.0 ± 19.6	454.3 ± 14.1	0.812	396.8 ± 16.1‡,§	378.7 ± 12.8‡,§	0.276
Hepatic insulin resistance	716 ± 84	1,159 ± 97	0.001	1,314 ± 112†	1,991 ± 183‡,§	0.002
Hepatic extraction (%)	76 ± 5	73 ± 4	0.893	69 ± 5	75 ± 4	0.375
Insulinogenic index ($\mu U_{insulin} \cdot g^{-1}_{glucose}$)	190.7 ± 17.1	98.7 ± 10.7	0.0001	130.3 ± 13.6‡	62.1 ± 10.1*,†	0.0001
Cp-genic index ($ng_{C\text{-}pep} \cdot g^{-1}_{glucose}$)	687.3 ± 46.4	536.2 ± 29.0‡	0.004	539.1 ± 53.5‡	402.6 ± 23.6*,†	0.009
Disposition index ($\mu U_{insulin} \cdot g^{-1}_{glucose} \cdot mL^{-1} \cdot m^{-2}$)	87,109 ± 3,506	46,015 ± 2,309	0.0002	51,749 ± 3,983‡	25,720 ± 2,776‡,§	0.0001
Adaptation index ($ng_{C\text{-}pep} \cdot g^{-1}_{glucose} \cdot mL^{-1} \cdot m^{-2}$)	316,498 ± 27,191	249,528 ± 11,192	0.008	217,586 ± 28,763*	152,715 ± 11,689‡,§	0.004
Incretin effect (%)	75.38 ± 4.01	55.08 ± 2.01	0.0005	65.87 ± 3.11‡	50.81 ± 2.29*,†	0.008

Data are expressed as the mean ± standard error of the mean. Insulinogenic index (IGI) = Δi30/Δg30. Cp-genic index (CGI) = Δcp30/Δg30. Disposition index = OGIS · IGI. Adaptation index = OGIS · CGI.

*$P < 0.05$ versus control TCF7L2 CT/TT.

†$P < 0.05$ versus control TCF7L2 CC.

‡$P < 0.001$ versus control TCF7L2 CT/TT.

§$P < 0.001$ versus control TCF7L2 CC.

controls ($P = 0.0001$), and homozygous T/T carriers were 7% in NAFLD versus 4% in controls ($P = 0.851$). TCF7L2 genotype distribution was in the Hardy-Weinberg equilibrium and was consistent with previous reports in Caucasians.[8-10] Due to the low prevalence of TCF T/T and MTP T/T genotypes and to the comparable features, they were grouped together with heterozygous carriers for comparisons.

High-density lipoprotein cholesterol levels were lower and diastolic blood pressure was higher in NAFLD than in controls, and within each group they differed between TCF7L2 TT/CT and TCF7L2 CC carriers. Females had lower fasting adiponectin and higher high-density lipoprotein cholesterol levels than males in each group, but there was no sex difference in any other fasting or postprandial parameter (see below).

Adopting the ATP III criteria for definition of the metabolic syndrome, 42% NAFLD TCF7L2 CC and 46% NAFLD TCF7L2 TT/CT had the whole picture of the metabolic syndrome (at least three criteria met). For details on liver histology in patients with NASH, see the Supplementary Material and Methods online.

Alimentary Record. There was no difference in daily total energy and macronutrient intake between patients with NAFLD and controls (2,501 ± 157 versus 2,545 ± 171 kcal [$P = 0.902$]; carbohydrate, 49 ± 3 versus 50 ± 3% kcal [$P = 0.802$]; protein, 19 ± 4 versus 19 ± 3 % kcal [$P = 0.697$]; fat, 32 ± 2 versus 31 ± 2% kcal [$P = 0.903$]).

Patients with NAFLD consumed more saturated fat and less polyunsaturated fat than controls when expressed as both percent total calories and percent total fat intake as previously reported[29] (saturated fatty acids, 13.1 ± 0.6 versus 10.4 ± 0.3% total kcal [$P = 0.0003$]; polyunsaturated fatty acids, 3.4 ± 0.3 versus 5.3 ± 0.3% total kcal [$P = 0.0002$]). Polyunsaturated to saturated fat ratio was also lower in NAFLD than in controls (0.27 ± 0.04 versus 0.50 ± 0.04 [$P = 0.0001$]).

NAFLD patients also had a lower daily vitamin A (605 ± 83 versus 1,127 ± 112 mg [$P = 0.007$]), vitamin C (114 ± 11 versus 158 ± 11 mg [$P = 0.031$]), and vitamin E (5.4 ± 0.9 versus 9.2 ± 0.4 mg [$P = 0.0006$]) intake. Daily alcohol intake was similar in the two groups (10 ± 2 versus 11 ± 2 g [$P = 0.923$]). There was no difference in daily antioxidant intake or in any other macronutrient/micronutrient intake between TCF7L2 genotypes within the NAFLD and the control group.

OGTT-Derived Indexes of Glucose Homeostasis. OGTT-derived indexes of glucose homeostasis are represented in Table 2. Hepatic insulin resistance and β-cell dysfunction were higher and the incretin effect was lower in TCF CT/TT than in TCF CC carriers in patients and controls.

Oral Fat Tolerance Test. Apolipoprotein E genotype distribution did not differ between NASH and controls and between different TCF genotypes in NASH. Postprandial plasma Tg, LDL-conjugated diene, and VLDL subfraction responses of both intestinal and he-

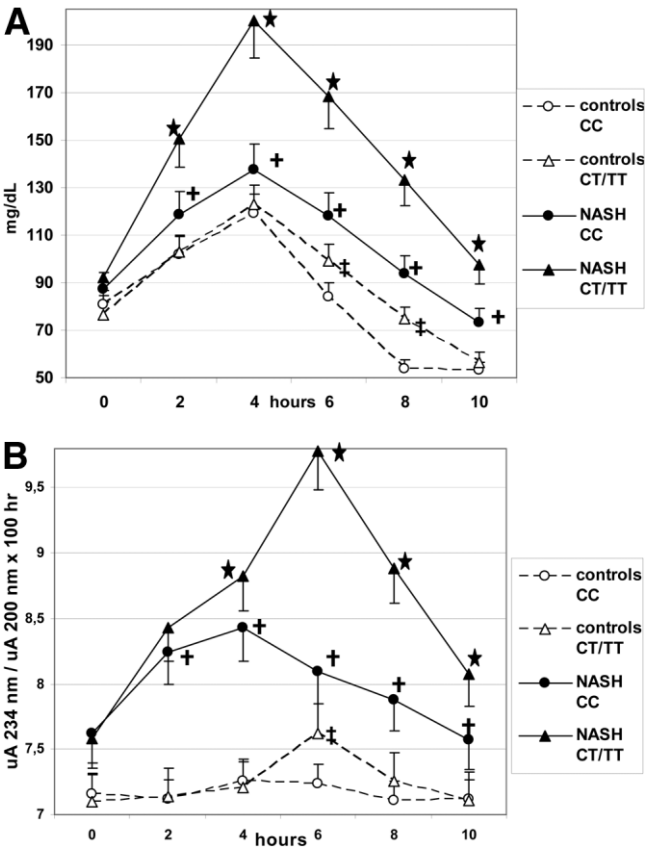

Fig. 1. Postprandial (A) Tg and (B) LDL-conjugated diene levels following a high-fat load in patients and controls, grouped according to TCF7L2 C/T genotype. Data are presented as the mean ± standard error of the mean. *$P < 0.05$ versus NASH TCF7L2 CC. †$P < 0.05$ versus controls TCF7L2 CT/TT. ‡$P < 0.05$ versus controls TCF7L2 CC.

patic origin were higher in NASH than in controls. Within patients and controls, plasma Tg, oxLDL, and VLDL subfraction responses were higher in TCF7L2 CT/TT than in CC carriers (Fig. 1A,B; Table 3). LDL cholesterol did not change throughout the test (data not shown).

Fasting plasma resistin was comparable between patients and controls and increased significantly postprandially in both groups. The increase was higher in NASH than in controls, and within each group it was higher in the TCF TT/CT genotype (Table 3; Fig. 2A). Fasting plasma adiponectin was lower in patients than in controls and significantly rose in controls, whereas it decreased in NASH (Fig. 2B). In controls, postprandial adiponectin increase was significantly smaller in TCF7L2 CT/TT than in TCF7L2 CC carriers. In NASH, postprandial adiponectin fell deeper in TCF7L2 CT/TT and increased nonsignificantly in TCF7L2 CC carriers (Fig. 2B; Table 3). Fasting and postprandial plasma CK-18 fragments were higher in NASH than in controls, and within each group they rose significantly higher in TCF TT/CT than in TCF CC genotype (Table 2; Fig. 2C).

Correlative Analysis. Main correlations between anthropometric, metabolic, and dietary parameters in NASH are shown in Supplementary Table 2 online. The results of multiple regression analysis are as follows:

- OGIS was independently predicted by fasting adiponectin ($\beta = 0.39$; $P = 0.02$).
- Hepatic insulin resistance index was predicted by IAUC adiponectin ($\beta = -0.48$; $P = 0.006$) and IAUC resistin ($\beta = 0.42$; $P = 0.009$).
- Insulinogenic index was predicted only by TCF7L2 polymorphism ($\beta = -0.42$; $P = 0.009$) and IAUC LDL-conjugated dienes ($\beta = -0.40$; $P = 0.01$).
- Disposition index was predicted by TCF7L2 polymorphism ($\beta = -0.50$; $P = 0.003$) and IAUC LDL-conjugated dienes ($\beta = -0.43$; $P = 0.008$).
- CP-genic index correlated with TCF7L2 polymorphism ($\beta = -0.40$; $P = 0.010$) and IAUC LDL-conjugated dienes ($\beta = -0.42$; $P = 0.009$).
- Adaptation index was predicted by TCF7L2 ($\beta = -0.43$; $P = 0.009$) and IAUC LDL-conjugated dienes ($\beta = -0.44$; $P = 0.008$).
- Incretin effect (the effectiveness of ingested glucose on insulin secretion) was independently predicted by TCF7L2 ($\beta = -0.42$; $P = 0.009$).
- TCF7L2 polymorphism was also an independent predictor of IAUC-Tg ($\beta = 0.49$; $P = 0.004$), IAUC VLDL1 apoB48 ($\beta = 0.44$; $P = 0.008$), and IAUC VLDL1 apoB100 ($\beta = 0.42$; $P = 0.009$).
- IAUC LDL-conjugated dienes were predicted by IAUC VLDL1 apoB48 ($\beta = 0.45$; $P = 0.007$) and IAUC adiponectin ($\beta = -0.51$; $P = 0.002$).
- IAUC adiponectin was predicted by TCF7L2 polymorphism ($\beta = -0.42$; $P = 0.009$) and dietary polyunsaturated fatty acid intake, expressed as %E ($\beta = 0.43$; $P = 0.008$).
- IAUC resistin was predicted by TCF7L2 ($\beta = 0.49$; $P = 0.004$).
- IAUC CK-18 was predicted by fasting CK-18 ($\beta = 0.40$; $P = 0.01$), IAUC adiponectin ($\beta = -0.45$; $P = 0.007$), and IAUC oxLDL ($\beta = 0.44$; $P = 0.008$).

The main determinants of liver histology are as follows:

- Severe hepatic steatosis was predicted by TCF7L2 (odds ratio [OR] = 2.2, confidence interval [CI] 1.7-4.9; $P = 0.008$), postprandial adiponectin decrease (for each quartile: OR = 2.0, CI 1.6-4.3; $P = 0.010$), and IAUC VLDL1 apoB48 (OR = 2.0, CI 1.6-4.3; $P = 0.010$).
- Necroinflammatory grade 3 was predicted by IAUC CK-18 (OR 2.0, CI 1.5-3.9; $P = 0.011$), postprandial adiponectin decrease (for each quartile: OR 1.9, CI 1.3-4.5; $P = 0.020$) and IAUC LDL-coniugated dienes (OR 2.1, CI 1.7-4.0; $P = 0.009$).

Table 3. Oral Fat Load Parameters of Patients with NASH According to TCF7L2 rs7903146C/T Polymorphism

	Control			NASH		
	TCF CC (n = 17)	TCF CT/TT (n = 17)	*P* Value	TCFCC (n = 13)	TCF TT/CT (n = 21)	*P* Value
Fasting Tg (mg/dL)	81 ± 7	78 ± 6	0.747	87 ± 11	92 ± 10	0.731
IAUC Tg (mg/dL × hours)	48 ± 20	155 ± 31	0.020	214 ± 38*,†	636 ± 42‡,§	0.0001
Fasting VLDL1-apoB48 (mg/dL)	2.28 ± 0.46	2.52 ± 0.51	0.691	5.84 ± 1.53*,†	6.81 ± 1.04‡,§	0.602
IAUC VLDL1-apoB48 (mg/dL × hours)	1.84 ± 0.41	5.04 ± 0.84	0.0006	8.61 ± 2.69‡,§	23.17 ± 2.56‡,§	0.007
Fasting VLDL2-apoB48 (mg/dL)	0.86 ± 0.280.23	0.61 ± 0.24	0.173	1.68 ± 0.42*,†	1.96 ± 0.50‡,§	0.369
IAUC VLDL2-apoB48 (mg/dL × hours)	2.09 ± 0.28	3.31 ± 0.73	0.10	2.36 ± 0.41*	6.94 ± 1.40†	0.002
Fasting VLDL1-apoB100 (mg/dL)	2.52 ± 0.51	2.28 ± 0.46	0.691	6.32 ± 1.26	6.20 ± 1.76	0.791
IAUC VLDL1-apoB100 (mg/dL × hours)	2.07 ± 0.49	5.34 ± 1.07	0.018	9.38 ± 1.91*,‡	24.79 ± 3.68‡,§	0.001
FastingVLDL2apoB100 (mg/dL)	1.69 ± 0.51	1.81 ± 0.28	0.340	3.41 ± 0.55*,†	4.28 ± 0.61*,§	0.508
IAUC VLDL2-apoB100 (mg/dL × hours)	1.85 ± 0.59	3.82 ± 1.65	0.09	2.36 ± 1.81*	5.54 ± 1.40†	0.008
Fasting LDL-conjugated dienes (uA 234 nm/ uA 200 nm × 100)	6.77 ± 1.34	6.64 ± 1.61	0.917	8.81 ± 2.92	7.16 ± 3.07	0.314
IAUC LDL-conjugated dienes (uA 234 nm/ uA 200 nm × 100 × hours)	0.3 ± 0.2	1.8 ± 0.6	0.021	3.3 ± 1.6*,§	13.0 ± 2.4‡,§	0.0003
Fasting resistin (ng/mL)	3.15 ± 0.31	3.47 ± 0.29	0.682	3.72 ± 0.23	3.70 ± 0.22	0.754
IAUC resistin (ng/mL × hours)	0.29 ± 0.18	2.36 ± 0.59	0.009	4.39 ± 0.98*,§	9.17 ± 1.56‡,§	0.00003
Fasting adiponectin (ng/mL)	12,058 ± 2,816	11,406 ± 2,018	0.873	5,789 ± 1,129‡,§	6,141 ± 907‡,§	0.810
IAUC adiponectin (ng/mL × hours)	16,734 ± 2,125	8,712 ± 1,186	0.001	1,215 ± 837	−9,331 ± 1,431‡,§	0.00001
Fasting CK-18 (U/L)	92 ± 6	103 ± 7	0.242	246 ± 10*,§	291 ± 11‡,§	0.011
IAUC CK-18 (U/L × hours)	148 ± 16	208 ± 21	0.030	321 ± 25*,§	439 ± 31‡,§	0.007

Data are expressed as the mean ± standard error of the mean.
*$P < 0.05$ versus control TCF7L2 CT/TT.
†$P < 0.05$ versus control TCF7L2 CC.
‡$P < 0.001$ versus control TCF7L2 CT/TT.
§$P < 0.001$ versus control TCF7L2 CC.

- Stage 3 fibrosis was predicted by TCF7L2 polymorphism (OR 2.0, CI 1.6-4.1; $P = 0.010$) and IAUC LDL-conjugated dienes (OR 2.1, CI 1.7-4.3; $P = 0.009$).

Discussion

The main findings of this study are: (1) rs7903146C/T TCF7L2 polymorphism is associated with NAFLD; (2) TCF7L2 polymorphism modulates postprandial hepatocyte apoptosis, coupled with the unfavorable postprandial lipoprotein and adipokine profile induced by fat ingestion in NASH; and (3) TCF7L2 polymorphism impacts pancreatic β-cell function, incretin effect, and hepatic insulin resistance in NASH and controls.

A novel finding is the association of TCF7L2 polymorphism with the risk of fatty liver and with the severity of liver disease in NASH. Although this polymorphism seems directly associated with the severity of steatosis and fibrosis, its association with necroinflammation appears mediated by adiponectin and oxLDL responses to fat ingestion.

Mechanisms of action of TCF7L2 are poorly understood. TCF7L2 is a nuclear receptor for β-catenin, regulating the expression of different genes involved in cell proliferation, differentiation, and metabolism. Its polymorphisms have been recently linked to impaired insulin secretion and risk of diabetes, an effect possibly mediated by reduced incretin secretion and by a direct modulation of β-cell responsivity to glucose.[8,10] Our data link TCF7L2 polymorphism to liver disease in NAFLD.

TCF7L2 axis may induce liver injury in NAFLD by hepatic and extrahepatic mechanisms. In the liver, the β-catenin/TCF pathway regulates many key enzymes involved in cellular redox balance, including cytochrome P450 and glutathione *S*-transferase and directly modulates stellate cell activation and fibrogenesis.[34]

At the extrahepatic level, TCF7L2 is expressed in adipocytes, where its activation, as seen in at-risk genotypes, promotes a proinflammatory phenotype, favoring lipolysis, reducing adiponectin, and enhancing resistin gene transcription.[35] Furthermore, TCF modulates Tg metabolism and adipokine secretion in adipocytes by regulating peroxisome proliferator-activated receptor gamma, CCAAT/enhancer-binding protein-α, and lipoprotein lipase gene transcription, three key genes involved in adipogenesis and lipid metabolism.[36] In this context, it is interesting to note that peroxisome proliferator-activated receptor gamma agonists, currently proposed for the treatment of NAFLD, promote adipocyte differentiation by enhancing β-catenin degradation and inhibiting TCF activation.[35]

The postprandial increase in markers of hepatocyte apoptosis seen in our patients provides a novel proinflammatory mechanism linking dietary fat to NASH[5,6] (i.e.,

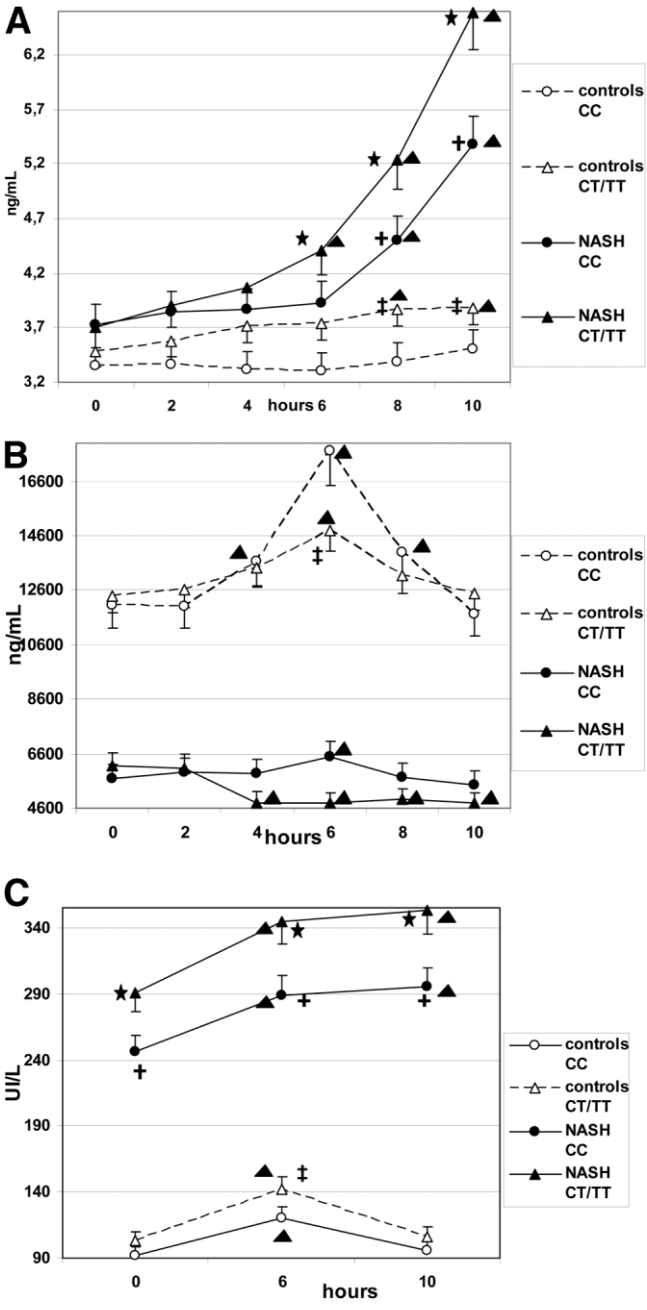

Fig. 2. Postprandial (A) resistin, (B) adiponectin, and (C) CK-18 following a high-fat load in patients and controls, grouped according to TCF7L2 C/T genotype. Data are presented as the mean ± standard error of the mean. Adiponectin levels were significantly higher in NASH than in controls at all time points. *$P < 0.05$ versus controls CC. †$P < 0.05$ versus controls TCF7L2 CT/TT. ‡$P < 0.05$ NASH GG versus controls TCF7L2 CC. ▲, $P < 0.05$ versus basal values.

acute adipokine imbalance induced by a high-fat meal). Although high-fat–induced nonalcoholic liver injury is a widely used experimental animal model of NASH,[5,6] data relating different nutrients to NAFLD are scarce and conflicting in humans. Kechagias et al.[37] showed that 1 week of fast-food–based hyperalimentation induced elevation in alanine aminotransferase levels and triglyceride content in healthy subjects. Such change was unrelated to caloric intake but tended to correlate with weight and fat mass gain, further suggesting adipose tissue–derived adipokines and/or fat-released free fatty acids may play a role in fatty liver. However, a clear dietary culprit for these changes could not be identified. We previously found that patients with NASH have a higher saturated fatty acid intake than healthy controls.[29] The association of the postprandial phase with the severity of liver histology and with circulating markers of hepatocyte apoptosis suggests postprandial lipemia may actually be harmful to the liver. Despite comparable fasting lipid, adipokine, and oxLDL levels, the lipid challenge evoked significant differences in lipoperoxidative markers, adiponectin, resistin and hepatocyte apoptosis between TCF genotypes, thus underlying the relevance of the postprandial metabolism for the pathogenesis of liver disease and associated metabolic disorders.[38-40] These data may provide a rationale for using lipid-lowering drugs in NASH, even in normolipidemic subjects.[12]

Glucose homeostasis was also assessed in our study to elucidate mechanisms linking fatty liver to the development of diabetes.[1] We found TCF7L2 polymorphism is associated with β-cell dysfunction in NASH as well, since this polymorphism independently predicted minimal model–derived parameters of β-cell dysfunction and incretin effect. Taken together, these findings suggest that TCF7L2 may impact β-cell function both directly through modulating β-cell response to glucose and indirectly by modulating incretin action or secretion (Fig. 3). If confirmed by larger studies, these findings might provide a pathophysiological basis for the use of incretin analogues in NASH.[4]

TCF polymorphism modulated hepatic insulin sensitivity, and TCF CT/TT had higher hepatic insulin resistance than TCF CC carriers in patients and controls. Coexistence of hepatic insulin resistance and steatosis is common, but underlying mechanisms are unclear and the severity of hepatic insulin resistance is often unrelated to the extent of steatosis. The association of postprandial, rather than fasting, adipokine levels with hepatic insulin resistance in NASH suggests that fat ingestion disturbs hepatic insulin sensitivity through dynamic adipokine imbalance (Fig. 3). Consistently, experimental evidence confirms that hepatic, rather than peripheral, insulin resistance closely correlates with acute changes in resistin and adiponectin levels.[41,42] Postprandially, the liver is flooded with nutrients and the balance between these two adipokines may be crucial for hepatic glucose homeostasis. One can speculate if circulating adiponectin increases to enhance lipid oxidation and Tg-rich lipoprotein catabolism, the liver does not accumulate Tg (controls); if adi-

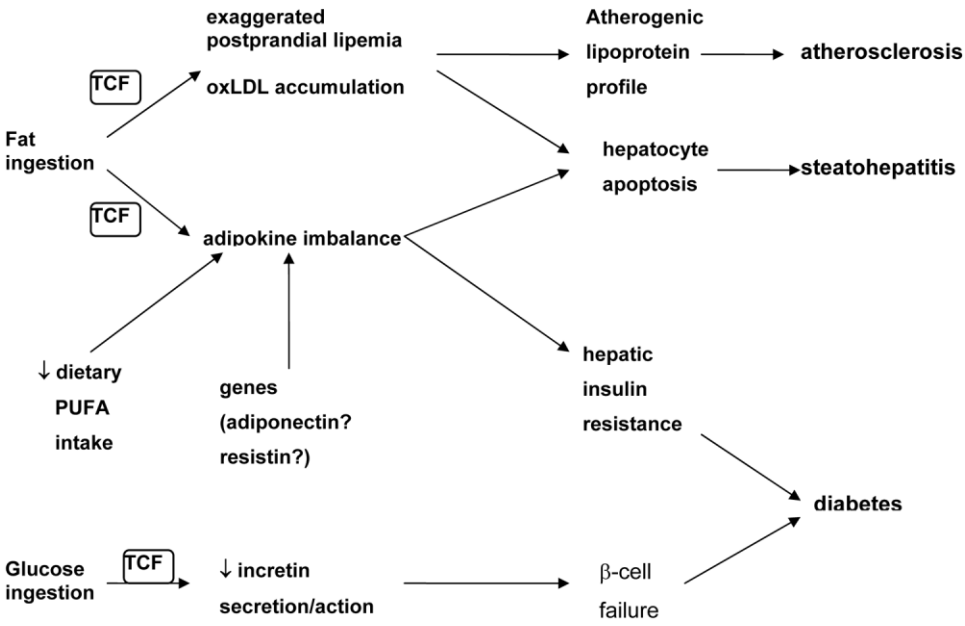

Fig. 3. Potential sites of action of TCF7L2 polymorphism in the pathogenesis of atherosclerosis, steatohepatitis, and diabetes after fat or glucose ingestion in NASH.

ponectin fails to increase, then fatty liver occurs. The type of fat in the background diet may modulate adipocyte adiponectin secretion differently in response to fat ingestion and may explain the differerent adiponectin response between patients and controls.[43]

The limitations of this study are its cross-sectional nature, which prevents any causal inference, and the small number of subjects. Our study subjects were predominantly male, and the applicability of our results to female sex need to be further tested.

In conclusion, TCF7L2 polymorphism predisposes to NAFLD and to a constellation of metabolic disorders involving glucose and lipoprotein homeostasis. A simple OGTT disclosed striking differences in β-cell function and hepatic insulin sensitivity, which may potentially direct more tailored interventions toward restoring hepatic insulin sensitivity and β-cell function in the rapidly growing therapeutic scenario of NAFLD. If our findings are prospectively confirmed in large cohort studies, assessing TCF polymorphism may help identify patients with NAFLD at higher hepato-cardio-metabolic risk who might benefit from improving postprandial lipid and glucose metabolism even in the presence of normal fasting values. Furthermore, in light of the multiple cellular events regulated by the TCF/Wnt pathway, a strategy aiming at modulating TCF activation may have several beneficial effects in NASH.

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