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Hypoadiponectinemia Predicts the Severity of Hepatic Fibrosis and Pancreatic Beta-Cell Dysfunction in Nondiabetic Nonobese Patients with Nonalcoholic Steatohepatitis

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OBJECTIVES: The relationships between the adipokines tumor necrosis factor (TNF)-α and adiponectin and the parameters of glucose homeostasis and severity of liver disease were assessed in nonobese nondiabetic subjects with nonalcoholic steatohepatitis (NASH).

METHODS: A frequently sampled intravenous glucose tolerance test, serum cytokine measurement, and 7-day alimentary record were performed in 20 biopsy-proven NASH patients and 45 age-, sex-, and BMI-matched controls (30 insulin sensitive and 15 insulin resistant).

RESULTS: Patients with NASH had impaired pancreatic β-cell function compared with both insulin-sensitive (adaptation index, AI: 97.7 ± 17.7 *vs* 307.4 ± 24.1 min^{-2} $mmol^{-1}$ L; $p = 0.00001$) and insulin-resistant (adaptation index, AI: 97.7 ± 17.7 *vs* 201.4 ± 41.1 min^{-2} $mmol^{-1}$ L; $p = 0.001$) controls. Serum adiponectin levels were also significantly lower in the NASH group than in the two control groups and correlated with adaptation index and with the severity of hepatic steatosis, necroinflammation, and fibrosis. When NASH patients were grouped according to the severity of histological liver damage, adiponectin was the only variable discriminating patients with higher necroinflammatory grade and fibrosis score from those with milder lesions.

CONCLUSIONS: β-cell secretory impairment is present in nonobese patients with NASH before glucose intolerance appears and may contribute to their increased risk for developing diabetes. Hypoadiponectinemia is a feature of NASH and may have a pathogenetic role in β-cell dysfunction and in hepatic necroinflammation and fibrosis, independently of insulin resistance, visceral fat accumulation, TNF-α axis activity, and dietary habits. Our findings provide further rationale for therapeutic approaches aimed at increasing adiponectin levels together with restoring β-cell function and insulin sensitivity.

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INTRODUCTION

Nonalcoholic steatohepatitis (NASH) is a chronic liver disease, histologically resembling alcoholic liver disease, encountered in individuals without significant alcohol consumption; it is part of a spectrum of liver damage, ranging from simple steatosis to advanced fibrosis and cirrhosis, named nonalcoholic fatty liver disease (NAFLD). NAFLD has become the most common chronic liver disease in Western countries, with a prevalence of 20% among adults in the United States (1). NASH is strongly associated with the metabolic syndrome, being present from early stages of the syndrome until overt type 2 diabetes mellitus has appeared, but the mechanism(s) underlying this association are not completely clear. In the NHANES III study, adults with NAFLD were more than twice as likely to have diabetes than those without NAFLD after adjustment for BMI, age, gender, and race (2); in two large, prospective cohort studies, high alanine aminotransferase levels predicted the subsequent development of type 2 diabetes mellitus independently of classical risk factors (3, 4).

Conversely, the presence of type 2 diabetes conveys a high risk for an aggressive outcome in NAFLD, doubling the risk for cirrhosis and increasing liver-related mortality 20-fold (5–7). The factor(s) responsible for the progression of metabolic and hepatic disease in NAFLD are poorly understood. Insulin

resistance is a hallmark of NASH, and current therapeutic approaches aim at improving insulin sensitivity in patients with overt obesity and diabetes (8), but no systematic evaluation of β-cell function in NASH is available and little is known about glucose homeostasis in NASH at earlier stages of metabolic disease, *i.e.*, before diabetes mellitus and obesity appear. An impairment in β-cell function predicts the development of diabetes mellitus in different populations (*i.e.*, older individuals and women with a history of gestational diabetes or polycystic ovary syndrome) independently of the degree of insulin resistance (9, 10). Furthermore, adipocyte-derived cytokines, or adipokines, in particular tumor necrosis factor (TNF-α) and adiponectin, have been recently implicated in the pathogenesis of type 2 diabetes mellitus and NASH, via their metabolic and pro-/anti-inflammatory activities (11–13).

This study assesses the relationships between two adipokines, TNF-α and adiponectin, and the severity of liver disease and factors regulating glucose homeostasis (*i.e.*, tissue insulin sensitivity, pancreatic β-cell function, and glucose effectiveness) in nonobese nondiabetic patients with biopsy-proven NASH.

MATERIALS AND METHODS

Subjects

Twenty patients (mean age $\pm$ SEM, 37 ± 3 yr, BMI 25.5 ± 0.7 kg/m^2) attending our Liver Unit during the years 2003–2004 were selected according to the following criteria: persistently (at least 12 months) elevated aminotransferases and ultrasonographic presence of bright liver without any other liver or biliary tract disease. Exclusion criteria were: a history of alcohol consumption >40 g/wk (assessed by a detailed inquiry and a validated questionnaire filled in daily for 1 wk by the patients); a body mass index (BMI) $\geq$30 kg/m^2 for males and $\geq$28 for females; positive serum markers of viral, autoimmune, or celiac disease; abnormal copper metabolism or thyroid function tests; a diagnosis of overt diabetes mellitus (fasting plasma glucose $\geq$126 mg/dL or $\geq$200 mg/dL at +2h on a standard oral glucose load, OGTT); serum total cholesterol $\geq$200 mg/dL, serum triglycerides $\geq$200 mg/dL; and exposure to occupational hepatotoxins or to drugs known to be steatogenic or to affect glucose metabolism. Mutations in the hemochromatosis HFE and TRF2 genes were detected in patients and controls using a single, multiplex amplification reaction and pre-made, ready-to-use teststrips (Nuclear Laser Medicine, Milan, Italy). Liver biopsy specimens were obtained from all patients and were blindly examined by a single pathologist (ED). Fatty infiltration, necroinflammation, and fibrosis were assessed as proposed by Brunt (14). Minimal histologic criteria for steatohepatitis were: steatosis involving al least 5% of hepatocytes, lobular inflammation, and zone 3 ballooning degeneration. Liver iron concentration (LIC) was determined on 2-mg dry weight tissue by atomic absorption spectroscopy. The hepatic iron index (HII) was obtained by dividing LIC (μmol/g) by age (years; normal range below 0.5).

The controls consisted of 30 healthy insulin-sensitive (defined by an insulin sensitivity index $S_I > 4.83 \times 10^{-4}$ min^{-1} μU^{-1} mL^{-1}) and 15 insulin-resistant (defined by an insulin sensitivity index $S_I < 4.83 \times 10^{-4}$ min^{-1} μU^{-1} mL^{-1}) individuals matched for age, sex, and BMI with normal liver enzymes and abdominal ultrasound scans (Table 1). The cut-off value (4.83×10^{-4} min^{-1} μU^{-1} mL^{-1}) was obtained by an analysis of the S_I of controls from this and other studies (8, 25). Patients and controls gave their consent to the study, which was conducted according to the Helsinki Declaration.

Alimentary Record

Patients and controls were instructed to fill in a 7-day dietary record during a 30-minute individual session with a trained nutritionist; a list of foods was designed, and for each item, different portion sizes were specified according to the EPIC study (15, 16). The recorded period included a complete week, and the record was collected within 1 wk of the glucose tolerance tests. 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 (17) and Food Composition Database for Epidemiological Study in Italy (18).

Cytokine Measurements

Serum TNF-α and adiponectin were measured by sandwich enzyme-linked immunosorbent assay (R&D System Europe Ltd, Abingdon, UK). For TNF-α, the kit has a sensitivity of 0.12 pg/mL in a 200-μL sample size and a range of 0.5–32 pg/mL. The intra- and inter-assay coefficients of variation were 5.9% and 12.6%, respectively. For human adiponectin, the kit has a sensitivity of 0.25 pg/mL in a 50-μL sample size and a range of 3.9–250 ng/mL. The intra- and inter-assay coefficients of variation were 3.4% and 5.8%, respectively. All samples were diluted by 1/100.

Oral Glucose Tolerance Test (OGTT)

After completion of the alimentary record and of baseline anthropometric measures and blood chemistry tests, patients and controls underwent a standard 75-g oral glucose tolerance test (OGTT), with measurement of plasma glucose and serum insulin concentrations at 0, 30, 60, 90, and 120 min.

Frequently Sampled Intravenous Glucose Tolerance Test (FSIGT)

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. Data analysis by the minimal model technique yielded the following parameters of glucose homeostasis (19, 20): glucose tolerance index (K_G), which represents the rate of disappearance of glucose from peripheral blood and is computed as the slope of the logarithm of the glucose concentration values between 12 and 40 min; insulin sensitivity index (S_I), which describes the ability of tissues to dispose of glucose under the action of insulin; and glucose

Table 1. Baseline Characteristics of Patients with NASH and Controls

	NASH (n = 20)	Insulin-Resistant Controls (n = 15)	Insulin-Sensitive Controls (n = 30)	P NASH *vs* Healthy
Age (yr)	37 ± 3	40 ± 3	36 ± 3	0.822
Sex (M/F)	19/1	13/2	28/2	0.999
BMI (kg/m^2)	25.5 ± 0.7	25.3 ± 0.6	25.3 ± 0.5	0.812
Family history of Type 2 diabetes (n° subjects)	3	4	4	0.631
Smokers (n° subjects)	4	3	6	0.999
Waist (cm)	90 ± 3	91 ± 3	87 ± 2	0.390
Systolic blood pressure (mmHg)	131 ± 3	130 ± 3	127 ± 3	0.370
Diastolic blood pressure (mmHg)	90 ± 2	89 ± 2†	77 ± 1	0.0001
Triglycerides (mg/dL)*	99 ± 13	103 ± 17	76 ± 6	0.080
Total cholesterol (mg/dL)†	175 ± 10	160 ± 12	169 ± 6	0.471
HDL cholesterol (mg/dL)†	47 ± 2	44 ± 3†	56 ± 4	0.089
LDL cholesterol (mg/dL)†	107 ± 10	105 ± 10	102 ± 5	0.444
Uric acid (mg/dL)†	6.18 ± 0.29	6.21 ± 0.35†	5.26 ± 0.22	0.013
Glucose (mg/dL)	92 ± 3	93 ± 3	86 ± 2	0.104
Insulin (μU/mL)	13.7 ± 2.1	12.1 ± 2.3†	7.2 ± 1.1	0.004
Albumin (g/dL)	4.8 ± 0.1	4.8 ± 0.1	5.0 ± 0.1	0.212
AST (U/L)	37 ± 3	25 ± 4*	26 ± 3	0.026
ALT (U/L)	87 ± 8	35 ± 4*	33 ± 4	0.0001
GGT (U/L)	87 ± 18	38 ± 8*	41 ± 4	0.006
ALP (U/L)	83 ± 8	50 ± 8	52 ± 7	0.011
HFE mutation (H63D) heterozygotes (n° subjects)	4	1	4	0.703
Serum iron (μg/dL)	97 ± 4	99 ± 4	88 ± 4	0.146
Ferritin (μg/L)	171 ± 21	140 ± 31	135 ± 20	0.227
Transferrin (% sat)	33 ± 1	30 ± 3	29 ± 2	0.131
TNF-α (pg/mL)	1.05 ± 0.14	1.08 ± 0.16	0.99 ± 0.08	0.525
Adiponectin (ng/mL)	4,378 ± 434	6,780 ± 511*,†	9,850 ± 940	0.00001

Data are presented as mean ± SEM.
*$p < 0.05$ *versus* NASH.
$^\dagger p < 0.05$ *versus* healthy controls.

effectiveness (S_G), which represents the ability of glucose *per se* to mediate its own uptake by tissues by mass action and to suppress endogenous glucose production independently of dynamic insulin action (19). Advantages and limitations of this technique have been extensively described in previous publications (20). Insulin secretion and liver degradation were described by: acute insulin response to glucose bolus (AIR_G), calculated by averaging the incremental concentration of insulin between 3 and 10 min after glucose injection; β-cell sensitivities to glucose (ϕ_1 and ϕ_2), *i.e.*, the ability of glucose to stimulate early (first-phase) and delayed (second-phase) C-peptide secretion; and average hepatic insulin extraction (Hmean), *i.e.*, the ability of the liver to extract insulin from the portal blood. The overall metabolic status was described by two indexes, which relate β-cell insulin secretion and post-hepatic delivery to insulin resistance: the disposition index (DI), calculated by multiplying S_I by AIR_G (21), and the adaptation index (AI), calculated by multiplying S_I by ϕ_1 (22). These indexes describe the ability of the system to compensate for changes (*i.e.*, a reduction) in insulin sensitivity by adapting (*i.e.*, increasing) insulin secretion, and represent integrated markers of β-cell function (23).

Statistical Analyses

Data are expressed as mean ± SEM. Differences between groups were analyzed by analysis of variance (ANOVA), followed by Student-Neuman-Keuls test, when variables were normally distributed; otherwise the Mann-Whitney test was used. Normality was evaluated by the Shapiro-Wilk test. The Chi-square test or Fisher's exact test was used to compare categorical variables, as appropriate. The Spearman rank correlation coefficient was used to estimate the relationship between different histological, anthropometric, dietary, and biochemical variables. Multiple regression analysis was applied when multiple associations were detected, after log transformation of skewed data. Differences were considered statistically significant if $p < 0.05$.

RESULTS

Baseline Parameters

Baseline features of NASH patients and controls are reported in Table 1. Patients and insulin-resistant controls had higher mean diastolic pressures, fasting serum insulin levels, and uric acid levels than insulin-sensitive controls.

There was no significant difference in the number of smokers among the three groups (4 patients and 6 controls). Four patients and five controls were heterozygous carriers of the H63D mutation of the HFE gene (p = N.S.).

Adopting the Adult Treatment Panel III criteria for the clinical definition of the metabolic syndrome (24), 15 patients had hypertension (systolic/diastolic blood pressure 130/85 mmHg), one was hypertriglyceridemic (fasting plasma triglycerides 150 mg/dL), five had low plasma

Table 2. Minimal Model Parameters of Patients with NASH and Controls

	NASH (n = 20)	Insulin-Resistant Controls (n = 15)	Insulin-Sensitive Controls (n = 30)	P NASH *vs* Healthy
S_I (10^{-4} min^{-1} μU^{-1} mL^{-1})	3.93 ± 0.55	3.19 ± 0.69†	7.91 ± 0.61	0.0001
S_G ($min^{-1)}$	0.022 ± 0.003	0.020 ± 0.002	0.025 ± 0.001	0.277
ϕ_1 (pmol $min^{-1}mg^{-1}dL$)	76.05 ± 9.93	114.01 ± 25.14	125.50 ± 10.52	0.003
ϕ_2 (pmol $min^{-2}mg^{-1}dL$)	0.051 ± 0.014	0.050 ± 0.004	0.048 ± 0.006	0.826
AIR_G (μU/mL)	72.2 ± 16.6	66.9 ± 18.7	64.6 ± 6.95	0.660
DI ($min^{-1)}$	228.2 ± 45.6	321.7 ± 51.7*,†	488.5 ± 43.1	0.0001
AI (min^{-2} $mmol^{-1}$ L)	97.7 ± 17.7	201.4 ± 41.1*,†	307.4 ± 24.1	0.00001
Hmean (%)	69 ± 4	78 ± 4	72 ± 4	0.544
K_G (%/min)	1.71 ± 0.23	2.01 ± 0.36	2.18 ± 0.17	0.090

Data are presented as mean ± SEM.
*$p < 0.05$ *versus* NASH.
†$p < 0.05$ *versus* healthy controls.

HDL-cholesterol (HDL-C <40 mg/dL in men and <50 mg/dL in women), five had impaired glucose regulation (one had impaired fasting glycemia and five had impaired glucose tolerance on the OGTT; see below), and one had abdominal obesity (waist circumference >102 cm in men and >88 cm in women). Only four patients had the whole picture of the metabolic syndrome (at least three criteria met), and the remaining patients had only one (six patients) or two (10 patients) clinical features of the syndrome.

Cytokine Measurements

Serum adiponectin levels were significantly lower in patients with NASH than in insulin-resistant (4,378 ± 434 *vs* 6,780 ± 511 ng/mL; $p = 0.001$) and insulin-sensitive controls (4,378 ± 434 *vs* 9,850 ± 940 ng/mL; $p = 0.00001$), while there was no difference in serum TNF-α among the three groups.

Histopathology

Liver biopsy specimens were compatible with a diagnosis of NASH in all 20 patients: fatty infiltration was mild (involving 5–33% of hepatocytes) in 7 patients, moderate (33–66% of hepatocytes) in 6 patients, and severe (>66% of hepatocytes involved) in 7 patients. Necroinflammatory activity was grade 1 in 8 patients, grade 2 in 6 patients, and grade 3 in 6 patients. Fibrosis was stage 0 in 9 patients, stage 1 in 2 patients, stage 2 in 4 patients, and stage 3 in 5 patients; cirrhotic changes were absent in our patients. Liver iron concentration was 20 ± 2 μmol/g dry weight and hepatic iron index was 0.64 ± 0.03.

Alimentary Record

The daily total energy and macronutrient intakes of patients with NASH and controls were similar: total calories: 2,510 ± 123 *versus* 2,498 ± 148 kcal, $p = 0.898$; carbohydrate: 50 ± 2 *versus* 48 ± 2% kcal, $p = 0.598$; protein: 17 ± 3 *versus* 19 ± 2% kcal, $p = 0.568$; fat: 33 ± 2 *versus* 32 ± 1% kcal, $p = 0.673$. Patients with NASH consumed a diet richer in saturated fat and poorer in polyunsaturated fat than controls, when expressed as both the percentage of total calories and the percentage of total fat intake, as previously reported (25): SFA: 13.5 ± 0.8 *versus* 9.1 ± 0.5%% tot kcal, $p = 0.000$; PUFA: 3.5 ± 0.3 *versus* 5.0 ± 0.4% tot kcal, $p = 0.000$. The polyunsaturated to saturated fat ratio was also significantly lower in the NASH group (P/S ratio: 0. 24 ± 0.03 *vs* 0.47 ± 0.03, $p = 0.0002$).

Daily cholesterol intake was higher in patients than in controls: 506 ± 28 *versus* 410 ± 28 mg/d ($p = 0.002$). NASH patients also had a significantly lower daily intake of the antioxidant vitamin E (5.4 ± 0.5 *vs* 8.6 ± 0.6 mg, $p = 0.0001$), while there was no difference in the intakes of vitamin A and C. Dietary habits of controls were comparable to those of a large sample of healthy individuals of the Piedmont population, as assessed by a recent alimentary survey (16).

Oral Glucose Tolerance Test

No patient was classified as diabetic, five patients had impaired glucose tolerance (IGT) according to ADA Recommendations (26), and one patient had impaired fasting glycemia (fasting plasma glucose ≥110 mg/dL but <126 mg/dL).

Minimal Model Parameters

Patients showed a lower glucose tolerance (K_G) than controls, although the difference was not statistically significant (Table 2). S_I was markedly lower in patients than in insulin-sensitive controls, while S_G was similar. First-phase β-cell sensitivity to glucose, ϕ_1, was significantly lower in patients than in insulin-sensitive controls, though the absolute values of the insulin concentration in the early phase (AIR_G) were not different. Both the disposition index (DI) and adaptation index (AI) were significantly lower in patients than in the two control groups, with no difference between patients with a family history of type 2 diabetes mellitus and those without (not shown). Hepatic insulin extraction was similar in the three groups.

Normotolerant versus Impaired Glucose Tolerant Patients

Compared with healthy controls, patients with normal glucose tolerance (NGT; n = 15) displayed lower insulin sensitivity (S_I: 4.46 ± 0.55 *vs* 7.91 ± 0.61 10^{-4} min^{-1} μU^{-1} mL^{-1}; $p = 0.0001$), disposition index (DI: 259.9 ± 48.9 *vs* 488.5 ± 43.1 min^{-1}, $p = 0.002$), and adaptation index (AI: 111.1

± 17.7 *vs* 307.4 ± 24.1 pmol $min^{-1}mg^{-1}$ dL; $p = 0.0001$), while there was no difference in any other anthropometrical, biochemical, dietary, or histological parameters.

NGT patients were not significantly different from IGT patients in S_I (4.46 ± 0.55 *vs* 2.34 ± 0.40 10^{-4} min^{-1} μU^{-1} mL^{-1}; $p = 0.070$) or adaptation index (AI: 111.1 ± 17.7 *vs* 57.4 ± 10.1 pmol $min^{-1}mg^{-1}$ dL; $p = 0.084$), or in any of the other parameters.

Correlations Between Anthropometric, Metabolic, and Histological Parameters

Insulin sensitivity index (S_I) correlated with waist circumference ($r_s = –0.53$; $p = 0.014$), serum TNF-α ($r_s = –0.49$; $p = 0.027$), and saturated fat intake expressed as a percentage of calories ($r_s = –0.59$; $p = 0.005$). On multiple regression analysis, only saturated fat intake ($\beta = –0.54$; $p = 0.009$) and waist circumference ($\beta = –0.49$; $p = 0.010$) independently predicted changes in insulin sensitivity. Adaptation index (AI) correlated with serum adiponectin ($r_s = 0.60$; $p = 0.005$), serum TNF-α ($r_s = –0.47$; $p = 0.038$), ALT levels ($r_s = –0.45$; $p = 0.047$), and liver fatty infiltration ($r_s = –0.59$; $p = 0.008$), but not with any other variable. On multiple regression analysis, only serum adiponectin ($\beta = 0.48$; $p = 0.031$) and serum TNF-α ($\beta = –0.42$; $p = 0.033$) independently predicted changes in adaptation index.

Liver fatty infiltration, expressed as the percentage of hepatocytes involved, correlated with adiponectin levels ($r_s = –0.60$; $p = 0.005$), adaptation index ($r_s = –0.59$; $p = 0.008$), and waist circumference ($r_s = –0.50$; $p = 0.025$), but not with other parameters. On multiple regression analysis, only serum adiponectin levels were independently associated with liver fatty infiltration ($\beta = –0.50$; $p = 0.024$).

Inflammatory grade correlated with adiponectin levels ($r_s = –0.51$; $p = 0.029$) but not with any other parameter. Similarly, fibrosis score correlated with adiponectin levels ($r_s = –0.54$; $p = 0.012$) but not with steatosis, inflammation, or any other clinical or biochemical parameter.

To further explore the relationships between liver histology and the variables assessed, patients were grouped into 2 categories on the basis of the severity of inflammation: mild (grade 1; n = 8) and moderate-severe (grade 2–3; n = 12). The latter group had lower adiponectin levels than the former, but did not differ in any other anthropometrical, biochemical, or dietary parameter (the main characteristics of the two groups and of the controls are reported in Table 3). The mild inflammation group also had lower adiponectin levels than insulin-resistant controls: (5,061 ± 529 *vs* 6,780 ± 511 ng/mL; $p = 0.044$).

Patients were then divided into 2 subgroups on the basis of fibrosis score: mild-absent (score 0–1; n = 11) and

Table 3. Main Clinical, Biochemical, and Model Characteristics of NASH Patients with Histological Necroinflammatory Grade 1 and Grade 2 + 3 and of Controls

	Insulin-Sensitive Controls (n = 30)	NASH Grade 1 (n = 8)	NASH Grade 2–3 (n = 12)	P Grade 1 *vs* Grade 2–3
Age (yr)	36 ± 3	38 ± 3	37 ± 5	0.767
BMI (kg/m^2)	25.3 ± 0.5	25.4 ± 0.7	26.2 ± 0.9	0.274
Waist (cm)	87 ± 2	90 ± 2	92 ± 3	0.627
Impaired glucose tolerance (n° subjects)	0	1	4	0.603
Triglycerides (mg/dL)	76 ± 6	105 ± 21	92 ± 12	0.498
Total cholesterol (mg/dL)	169 ± 6	181 ± 10	166 ± 19	0.392
Uric acid (mg/dL)	5.26 ± 0.22	6.11 ± 0.39*	6.28 ± 0.15*	0.720
AST (U/L)	26 ± 15	37 ± 4†	37 ± 3	0.966
ALT (U/L)	33 ± 20	81 ± 13‡	96 ± 8‡	0.371
GGT (U/L)	41 ± 20	114 ± 25†	45 ± 17	0.096
ALP (U/L)	52 ± 31	94 ± 13	71 ± 8	0.135
Albumin (g/dL)	5.0 ± 0.1	4.8 ± 0.1	4.9 ± 0.1	0.184
Serum iron (μg/dL)	88 ± 4	101 ± 4	92 ± 6	0.227
Ferritin (μg/L)	135 ± 20	147 ± 23	210 ± 39	0.184
Transferrin (% sat)	29 ± 2	35 ± 4	29 ± 2	0.082
Liver iron concentration (μmol/g dry weight)	–	21 ± 4	15 ± 3	0.238
Hepatic iron index	–	0.64 ± 0.13	0.48 ± 0.05	0.204
TNF-α (pg/mL)	0.99 ± 0.08	0.94 ± 0.09	1.24 ± 0.3	0.292
Adiponectin (ng/mL)	9,850 ± 940	5,061 ± 529*	3,440 ± 286‡	0.012
S_I (10^{-4} × min^{-1} × μU^{-1} × mL^{-1})	7.91 ± 0.61	4.18 ± 0.81†	3.62 ± 0.75†	0.641
$\phi 1$ (pmol × $min^{-1}mg^{-1}dL$)	125.50 ± 10.52	83.94 ± 17.26*	69.59 ± 19.13*	0.516
DI ($min^{-1)}$)	488.5 ± 43.1	235.9 ± 45.7*	201.4 ± 72.9*	0.731
AI (min^{-2} × $mmol^{-1}$ × L)	307.4 ± 24.1	120.4 ± 27.4‡	80.1 ± 38.2‡	0.450
Hmean (%).	72 ± 4	77 ± 6	61 ± 7	0.124
S_G ($min^{-1)}$)	0.025 ± 0.001	0.023 ± 0.001	0.016 ± 0.002	0.101
K_G (%/min)	2.18 ± 0.17	1.73 ± 0.21	1.60 ± 0.14	0.603

Data are presented as mean ± SEM.
*$p < 0.05$ *versus* controls.
†$p < 0.01$ *versus* controls.
‡$p < 0.001$ *versus* controls.

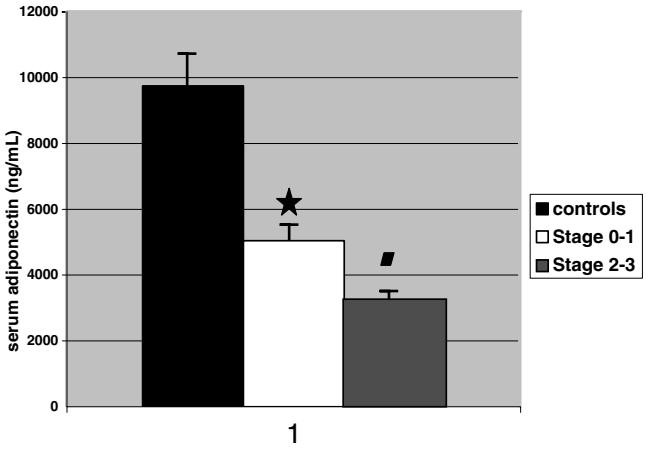

Figure 1. Serum adiponectin levels of NASH patients, according to fibrosis stage, and of insulin-sensitive controls: absent-mild fibrosis (stage 0–1), moderate-severe fibrosis (stage 2–3). Data are presented as mean ± SEM. ⋆$p < 0.01$ *versus* controls, ▰$p < 0.005$ *versus* NASH stage 0–1.

moderate-severe (score 2–3; n = 9). The moderate-severe fibrosis group had significantly lower adiponectin levels than the former (Fig. 1), but did not differ in any other anthropometrical, biochemical, or dietary parameter (the main characteristics are reported in Table 4). The mild-absent NASH group had lower adiponectin levels than insulin-resistant controls as well: 5,045 ± 433 *versus* 6,780 ± 511 ng/mL; $p = 0.021$.

DISCUSSION

The main determinants of glucose homeostasis were assessed in 20 patients with biopsy-proven NASH and were correlated with dietary habits, circulating levels of two adipokines, and liver histology. Nonobese nondiabetic patients were selected, since obesity and diabetes can, *per se*, affect β-cell function and serum adipokine levels (9, 27); furthermore, NAFLD is considered an early predictor of the future development of overt metabolic disorders in normal-weight glucose-tolerant individuals (28).

A novel finding of our study is that a marked β-cell secretory dysfunction was detectable in NASH long before glucose intolerance appeared, thus giving a strong pathophysiological basis, together with insulin resistance, for the increased risk for developing diabetes observed in this population (2, 3). In this context, we evaluated the disposition and adaptation indexes, which represent insulin secretion in relation to ambient insulin sensitivity and provide an accurate figure of pancreatic action (20–22). These indexes were lower in patients with NASH than in insulin-sensitive controls, and were also lower than those of matched insulin-resistant controls, indicating β-cell failure to compensate for decreased insulin sensitivity long before glucose intolerance appears in individuals at high risk for developing diabetes mellitus (9). The relevance of β-cell failure to the pathogenesis of diabetes in liver disease has been clearly demonstrated in cirrhosis, where improving insulin sensitivity by liver transplantation cures hepatogenous diabetes in only a portion of patients, while the others remain diabetic due to persistent β-cell secretory dysfunction (29).

Our finding highlights the importance of evaluating the effect of proposed therapies on β-cell function in NASH, since most of the current therapeutic approaches aim at improving insulin sensitivity, but their effects on islet cell secretion, as well as on the long-term progression of metabolic disease, are unknown in these patients.

Both genetic and acquired factors may have been responsible for β-cell dysfunction in our patients. It is well known that family members of diabetics have a reduced ability of β cells to compensate for impaired insulin sensitivity and are at a greater risk for developing diabetes; however, only three of our patients were first-degree relatives of diabetics, and their β-cell function did not differ from the other patients (not shown).

Impaired glucose tolerance may, *per se*, reduce β-cell function (21), but in our study, β-cell dysfunction was detectable in the 15 patients with normal glucose tolerance as well.

Alimentary factors, in particular a diet high in saturated fat, have been implicated in the pathogenesis of pancreatic β-cell dysfunction in cell cultures and animal models (30, 31). Although saturated fat intake was higher in patients than in controls, it correlated with the insulin sensitivity index, as previously reported (25), and not with the β-cell function indexes.

Our data suggest that the adipokines adiponectin and TNF-α may be implicated in the β-cell dysfunction seen in NASH. In fact, the adaptation index of our patients correlated with adiponectin and with TNF-α levels, the latter, however, being comparable to those of controls: a likely explanation is that, in the setting of hypoadiponectinemia, pancreatic islet cells become more susceptible to harmful factors such as visceral fat-released free fatty acids, TNF-α, and possibly saturated fat intake. Consistent with our data, adiponectin correlated with β-cell function indexes in healthy individuals and prevented pancreatic β-cell apoptosis in cell cultures by suppressing TNF-α and free fatty acid–induced nuclear factor (NF)-κB activation, a key mediator of inflammatory-induced gene transcription (32, 33). Furthermore, thiazolidinediones, a group of peroxisome proliferator-activated receptor (PPAR)-γ agonists that enhance insulin sensitivity and increase circulating adiponectin, were able to reduce islet triglyceride content and restore impaired glucose-stimulated insulin secretion in mice fed a high fat diet (34).

The adaptation index correlated with the severity of steatosis in patients with NASH. This finding may simply reflect the association of hypoadiponectinemia with both β-cell dysfunction and liver fatty infiltration or, alternatively, β-cell failure may directly contribute to liver fat accumulation in NASH: in healthy humans, acute hyperinsulinemia suppresses VLDL production both directly, by modulating intrahepatic VLDL assembly, and indirectly, by reducing plasma FFA availability (35), the latter ability being maintained in

Table 4. Main Clinical, Biochemical and Model Characteristics of NASH Patients with Fibrosis Stage 0–1, Fibrosis Stage 2–3, and of Controls

	Insulin-Sensitive Controls (n = 30)	NASH Stage 0–1 (n = 11)	NASH Stage 2–3 (n = 9)	P Stage 0–1 *vs* Stage 2–3
Age (yr)	36 ± 3	38 ± 3	36 ± 4	0.697
BMI (kg/m^2)	25.3 ± 0.5	25.4 ± 0.6	26.2 ± 1.1	0.461
Waist (cm)	87 ± 2	90 ± 2	92 ± 3	0.343
Impaired glucose tolerance (n° subjects)	0	2	3	0.999
Triglycerides (mg/dL)	76 ± 6	112 ± 15	75 ± 14	0.164
Total cholesterol (mg/dL)	169 ± 6	185 ± 9	156 ± 19	0.434
Uric acid (mg/dL)	5.26 ± 0.22	6.13 ± 0.31*	6.27 ± 0.38*	0.756
AST (U/L)	26 ± 15	37 ± 4	35 ± 3	0.983
ALT (U/L)	33 ± 20	80 ± 10*	97 ± 15*	0.367
GGT (U/L)	41 ± 20	115 ± 21*	66 ± 14	0.091
ALP (U/L)	52 ± 31	91 ± 11	66 ± 21	0.209
Albumin (g/dL)	5.0 ± 0.1	4.8 ± 0.1	4.9 ± 0.2	0.542
Serum iron (μg/dL)	88 ± 4	100 ± 4	88 ± 6	0.372
Ferritin (μg/L)	135 ± 20	141 ± 18	214 ± 46	0.129
Transferrin (% sat)	29 ± 2	35 ± 2	29 ± 3	0.094
Liver iron concentration (μmol/g dry weight)	–	19 ± 3	15 ± 3	0.363
Hepatic iron index	–	0.58 ± 0.11	0.48 ± 0.06	0.464
TNF-α (pg/mL)	0.99 ± 0.08	0.91 ± 0.08	1.30 ± 0.31	0.243
Adiponectin (ng/mL)	9,850 ± 940	5,045 ± 433†	3,234 ± 373‡	0.002
S_I ($10^{-4} \times min^{-1} \times \mu U^{-1} \times mL^{-1}$)	7.91 ± 0.61	4.22 ± 0.77†	3.50 ± 0.93†	0.558
ϕ1(pmol × min^{-1}mg^{-1}dL)	125.50 ± 10.52	82.72 ± 20.62*	70.97 ± 15.72*	0.667
DI (min^{-1})	488.5 ± 43.1	260.3 ± 73.64*	200.2 ± 70.3*	0.569
AI ($min^{-2} \times mmol^{-1} \times L$)	307.4 ± 24.1	125.6 ± 24.9‡	80.8 ± 41.4†	0.342
Hmean (%)	72 ± 4	78 ± 4	64 ± 8	0.115
S_G (min^{-1})	0.025 ± 0.001	0.023 ± 0.005	0.016 ± 0.002	0.165
K_G (%/min)	2.18 ± 0.17	1.73 ± 0.21	1.54 ± 0.17	0.999

Data are presented as mean ± SEM.
*$p < 0.05$ *versus* controls.
†$p < 0.01$ *versus* controls.
‡$p < 0.001$ *versus* controls.

obesity (36) and in type 2 diabetes (37). In NAFLD, the suppressive effect of insulin on plasma FFA availability was maintained during the first 2 h of euglycemic hyperinsulinemic clamp, and was then progressively lost (38). A blunting of the early β-cell insulin secretion may thus enhance hepatic TG accumulation by increasing plasma FFA availability, particularly in the setting of the exaggerated postprandial triglyceride response seen in these patients (25).

The other novel finding of our study is the independent association of hypoadiponectinemia with the severity of fibrosis deposition in patients with NASH. Previous studies reported that hypoadiponectinemia was closely related to hepatic fat content in diabetic patients (39) and that adiponectin delivery alleviated steatosis and LPS-induced liver injury in animal models of fatty liver disease, through modulation of TNF-α and PPAR-α activity (40, 41). Recently, Hui *et al*. found that hypoadiponectinemia and insulin resistance independently predicted the severity of steatosis and necroinflammation in NAFLD, suggesting that low adiponectin levels may be a feature of NASH (13). We found that, while the severity of steatosis paralleled the degree of insulin resistance and hypoadiponectinemia, only adiponectin levels correlated inversely with the severity of necroinflammation and of fibrosis in NASH, independently of insulin resistance, visceral fat accumulation, serum TNF-α level, and dietary intake.

This finding agrees with the reported ability of adiponectin to attenuate carbon tetrachloride–induced liver fibrosis in mouse models (42). The suppressive effect of adiponectin on platelet-derived growth factor- and transforming growth factor-β1–induced proliferation and migration of cultured hepatic stellate cells provides the molecular basis for the antifibrotic effect of this novel adipokine (42).

Notably, the severity of hepatic steatosis and necroinflammation did not correlate with fibrosis. Unlike fatty infiltration and necroinflammation, which can be altered over a short period of time in relation to lifestyle changes (*i.e.*, diet, physical activity, hormonal status), fibrosis progression may occur over a much longer period in NASH. Adipokine levels fluctuate over time depending on the metabolic milieu as well. It is therefore not surprising that cytokine levels measured in this cross-sectional study correlated with the severity of histological lesions, but there was no correlation between steatosis necroinflammation and fibrosis. The finding that changes in the severity of steatosis and inflammation run an independent course from those of fibrosis has been reported in previous studies (43, 13).

Serum TNF-α levels of our patients were comparable to those of controls, although they correlated inversely with insulin sensitivity and β-cell function This finding disagrees in part with the existing data (13) and may suggest that the

TNF-α pathway contributes to liver damage only at a later stage or, alternatively, that serum TNF receptor 2 or tissue TNF-α mRNA expression may be more sensitive markers of TNF-α axis activation.

In conclusion, we found that β-cell dysfunction and hypoadiponectinemia are early features of NASH, appearing well before glucose intolerance and/or the full picture of the metabolic syndrome have developed and may contribute to the progression of metabolic and liver disease to diabetes and cirrhosis. Hypoadiponectinemia, in particular, may be a link between impaired glucose homeostasis and liver disease, thus providing the basis for the epidemiological association between the metabolic syndrome and NASH and for a therapeutic strategy aimed at increasing circulating adiponectin in this population. Other factors, such as increased TNF-α activity, may intervene later when overt obesity and glucose intolerance have appeared. Furthermore, the ability of adiponectin levels to discriminate mild from severe forms of necroinflammation and fibrosis may be useful in the clinical management of NAFLD, allowing the selection of patients at higher risk for progressive liver disease for liver biopsy and more aggressive treatments. Given that larger trials are needed to confirm the pathogenetic role of hypoadiponectinemia in both metabolic and liver disease and to clarify the cause (genetic and/or acquired) of hypoadiponectinemia, thiazolidinediones seem, at present, to be a rational therapeutic choice in these patients, given their ability to improve insulin sensitivity, restore β-cell secretory function, divert fat from abdominal and liver deposits, and increase serum adiponectin concentrations (44, 45).

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