Nonalcoholic Fatty Liver Disease

A Feature of the Metabolic Syndrome

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Insulin sensitivity (euglycemic clamp, insulin infusion rate: 40 mU · m^{-2} · min^{-1}) was studied in 30 subjects with biopsy-proven nonalcoholic fatty liver disease (NAFLD), normal glucose tolerance, and a BMI <30 kg/m^2. Of those 30 subjects, 9 had pure fatty liver and 21 had evidence of steatohepatitis. In addition, 10 patients with type 2 diabetes under good metabolic control and 10 healthy subjects were studied. Most NAFLD patients had central fat accumulation, increased triglycerides and uric acid, and low HDL cholesterol, irrespective of BMI. Glucose disposal during the clamp was reduced by nearly 50% in NAFLD patients, as well as in patients with normal body weight, to an extent similar to that of the type 2 diabetic patients. Basal free fatty acids were increased, whereas insulin-mediated suppression of lipolysis was less effective (−69% in NAFLD vs. −84% in control subjects; $P = 0.003$). Postabsorptive hepatic glucose production (HGP), measured by [6,6-$^2H_2$]glucose, was normal. In response to insulin infusion, HGP decreased by only 63% of basal in NAFLD vs. 84% in control subjects ($P = 0.002$). Compared with type 2 diabetic patients, NAFLD patients were characterized by lower basal HGP, but with similarly reduced insulin-mediated suppression of HGP. There was laboratory evidence of iron overload in many NAFLD patients, but clinical, histological, and biochemical data (including insulin sensitivity) were not correlated with iron status. Four subjects were heterozygous for mutation His63Asp of the HFE gene of familiar hemochromatosis. We concluded that NAFLD, in the presence of normoglycemia and normal or moderately increased body weight, is characterized by clinical and laboratory data similar to those found in diabetes and obesity. NAFLD may be considered an additional feature of the metabolic syndrome, with specific hepatic insulin resistance. *Diabetes* 50:1844–1850, 2001

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ALT, alanine transaminase; ANOVA, analysis of variance; AST, aspartate transaminase; FFA, free fatty acid; HGP, hepatic glucose production; HOMA, homeostasis model assessment; LBM, lean body mass; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OGTT, oral glucose tolerance test.

The presence of fatty liver in patients with type 2 diabetes and obesity has long been reported (1–4). It is usually considered an incidental pathologic finding, with scarce or no clinical significance.

Only recently did Ludwig et al. (5) identify a syndrome characterized by the association of fatty liver and lobular hepatitis and chronically elevated alanine aminotransferase plasma levels in patients with negligible alcohol intake. The syndrome is mainly associated with obesity (6,7), diabetes (6,8–10), and hyperdyslipemia (8–12), but a few patients are lean, have normal fasting glucose and glucose tolerance, and show no evidence of increased plasma lipids (10). Patients with fatty liver and hepatitis are identified as having nonalcoholic steatohepatitis (NASH). They are part of the broad spectrum of nonalcoholic fatty liver diseases (NAFLDs), which also include pure steatosis. The limits between NAFLD and NASH are set only by liver histology and cannot be predicted on clinical or laboratory grounds (13).

From a clinical point of view, a few patients have ongoing liver injury: ~50% of NASH patients develop liver fibrosis, 15% develop cirrhosis, and 3% may progress to terminal liver failure, requiring liver transplantation (14). In 15–50% of cases, liver fibrosis or cirrhosis may also be diagnosed at presentation (15).

Recent studies have pointed to hyperinsulinemia and insulin resistance as pathogenic factors in NAFLD. Using the homeostasis model assessment (HOMA) method (16), Marchesini et al. (17) showed that insulin resistance was the laboratory finding most closely associated with the presence of NAFLD in a large series of patients, irrespective of BMI, fat distribution, or glucose tolerance. Accordingly, NAFLD might represent another feature of the metabolic syndrome, with decreased insulin sensitivity being the common factor (18). The strong association of NAFLD with other features of the metabolic syndrome (obesity, central fat distribution, diabetes, dyslipidemia, hypertension, and atherosclerotic cardiovascular disease) supports this hypothesis (19).

The euglycemic clamp technique remains the gold standard for quantitatively measuring insulin sensitivity (20). When coupled with stable isotopes, it gives clues to the site of insulin resistance (hepatic versus peripheral tissues) (21). In this study, we measured insulin sensitivity by means of the clamp technique in a group of patients with NAFLD, and compared the results with values obtained in

TABLE 1
Anthropometric, clinical, and laboratory data in NAFLD patients, type 2 diabetic patients, and control subjects

	NAFLD patients	Type 2 diabetic patients	Control subjects
n	30	10	10
Sex (M/F)	24/6	8/2	8/2
Age (years)	41 ± 11 (24–64)*	56 ± 8 (45–67)	48 ± 14 (26–60)
Weight (kg)	81 ± 10 (65–96)	84 ± 10 (66–105)	73 ± 11 (57–94)
BMI (kg/m^2)	27 ± 2 (23–29)†	29 ± 4 (24–37)†	24 ± 2 (21–28)
Waist circumference (cm)	100 ± 8 (87–115)†	98 ± 8 (77–109)	85 ± 12 (73–100)
Hip circumference (cm)	106 ± 6 (94–117)	109 ± 10 (100–135)	101 ± 6 (90–109)
Waist-to-hip ratio	0.94 ± 0.06 (0.80-1.08)†	0.92 ± 0.09 (0.71–1.04)	0.84 ± 0.10 (0.71–0.95)
Fasting glucose (mmol/l)	5.2 ± 0.5 (4.4–6.0)*	6.3 ± 0.4 (5.7–7.1)†	5.0 ± 0.4 (4.4–5.7)
120-min OGTT glucose (mmol/l)	5.7 ± 0.9 (4.1–7.2)	Not determined	5.9 ± 0.8 (4.7–7.2)
Total cholesterol (mmol/l)	5.5 ± 1.1 (3.9–9.1)	5.0 ± 0.8 (4.0–6.5)	4.9 ± 0.9 (3.4–5.9)
HDL cholesterol (mmol/l)	1.1 ± 0.3 (0.7–2.1)†	1.0 ± 0.2 (0.7–1.4)†	1.5 ± 0.3 (1.0–2.1)
Triglycerides (mmol/l)	2.3 ± 1.0 (0.9–4.9)†	2.3 ± 1.2 (0.7–4.3)†	1.1 ± 0.4 (0.6–1.6)
Uric acid (μmol/l)	349 ± 64 (208–476)†	358 ± 73 (227–479)†	235 ± 59 (156–329)
Fasting insulin (pmol/l)	124 ± 58 (43–265)†	143 ± 59 (86–258)†	44 ± 20 (14–79)
Fasting C-peptide (pmol/ml)	1,080 ± 236 (719–1,548)†	900 ± 298 (525–1,548)†	677 ± 200 (498–1,161)
AST (units/l)	42 ± 16 (25–98)†*	19 ± 7 (12–29)	16 ± 5 (10–22)
ALT (units/l)	90 ± 40 (44–203)†*	29 ± 10 (15–44)	22 ± 7 (12–33)

Data are means ± SD (range). Normal AST and ALT values <40 units/l. *$P < 0.005$ vs. patients with type 2 diabetes; †$P < 0.005$ vs. control subjects.

normal subjects and in patients with type 2 diabetes under good metabolic control.

RESEARCH DESIGN AND METHODS

Patients. The study was carried out in 30 NAFLD patients consecutively admitted to our department for the evaluation of chronically elevated transaminase levels (alanine transaminases [ALTs] exceeding by twofold the upper normal values in two or more determinations). Their pertinent clinical and laboratory data are reported in Table 1. Before entering the study protocol, these patients were systematically screened for the following putative etiologic factors: hepatitis B and C, Ebstein-Barr virus infection, non–organ-specific autoantibodies, α_1-antitrypsin deficiency, and copper storage disease. Iron status was included in the protocol and will be presented as a result. Alcohol consumption was assessed by detailed history and laboratory markers (serum γ-glutamyl transpeptidase and mean corpuscular volume of erythrocytes); patients consuming even moderate alcohol amounts (>20 g/day) were excluded. In all cases, relatives were also interviewed to exclude surreptitious alcohol consumption. To rule out diabetes as a confounder, patients with fasting or glucose-stimulated hyperglycemia, indicative of impaired glucose regulation according to American Diabetes Association (cutoff value, 6.1 mmol/l) (22) or World Health Organization criteria (120-min blood glucose ≥7.8 mmol/l after a 75-g glucose load) (23) were excluded. Similarly, subjects with BMI ≥30 kg/m^2 were not considered, to exclude obesity as a confounding factor. No patient had clinical evidence of liver disease, and routine liver function tests were within normal limits in all cases. All patients were submitted to an ultrasound scan of the liver to confirm fatty deposition (24) and to a percutaneous liver biopsy.

A group of 10 patients with type 2 diabetes was also studied. Those patients were in fairly compensated conditions under diet and oral hypoglycemic agents (mean HbA_{1C}, 7.2 ± 0.7 %; range 6.0–8.1). Their fasting blood glucose at the time of the clamp study was <7.8 mmol/l. They were selected on the basis of a normal ultrasound liver scan, an age range similar to that of the NAFLD patients, and normal transaminase levels and liver function tests. The last dose of pharmacological treatment with oral hypoglycemic agents (metformin, up to 2,550 mg/day in overweight patients; gliclazide, up to 240 mg/day; or a combination of the two) was given 12 h before the study.

A third group of subjects with normal weight, fasting glucose and glucose tolerance, liver function tests, and transaminase levels served as control subjects.

Cigarette smoking was not considered. All subjects gave written informed consent to take part in the study, which was approved by the ethical committee of our institution.

Clinical records, anthropometrics, blood tests, and liver biopsy. On admission to day-hospital, all subjects were carefully interviewed to obtain a detailed social and family history, and anthropometric data were taken to the nearest half-centimeter (height, waist, and hip circumference) or half-kilogram (weight). BMI was calculated as a measure of overweightness and/or obesity, whereas waist circumference and waist-to-hip ratio were measured as indexes of splanchnic fat accumulation (25). To eliminate interobserver variation, all anthropometric measurements were made by the same skilled physician.

Blood tests included a routine biochemistry, an oral glucose tolerance test (OGTT; 75 g glucose dissolved in 200 ml water; not done in the diabetic group), and blood sampling at 30-min intervals over a 120-min period. In addition, NAFLD patients had a complete evaluation of iron status (serum iron, transferrin, and ferritin concentrations) and were tested for the presence of the three mutations—Cys282Tyr, His63Asp, and Ser65Cys (14 cases)—in the recently cloned hemochromatosis gene, HFE gene (26).

A liver biopsy was taken in NAFLD subjects under ultrasound control. All specimens were examined by the same experienced pathologist and scored for fat infiltration, fibrosis, and necroinflammation on a scale of 0–3, as proposed by Diehl et al. (12).

Euglycemic-hyperinsulinemic clamp study. Insulin sensitivity was measured by means of the euglycemic-hyperinsulinemic clamp, according to the method of DeFronzo et al. (20). Briefly, between 7:30 and 8:30 A.M., a catheter was placed in an antecubital vein for glucose/insulin infusion. A second catheter, inserted retrogradedly in the opposite hand and kept patent by continuous saline infusion, was used for blood sampling. The hand was warmed with a heating pad to obtain arterialized blood samples.

After an equilibration period, insulin was infused using a primed, continuous protocol. The continuous infusion was kept at a rate of 40 mU · m^{-2} · min^{-1} for 2 h. Blood samples were obtained at 5-min intervals for immediate determination of plasma glucose, using an automated glucose oxidase technique (Beckman Glucose Analyzer II; Beckman Instruments, Fullerton, CA). The results were used to titrate the infusion rate of a 20% glucose solution to prevent hypoglycemia and to maintain blood glucose at levels within ± 0.5 mmol/l of the fasting value or at a predetermined value of 5.0 mmol/l whenever glucose levels were higher. To achieve this value, blood glucose was allowed to decrease slowly within the first 30 min by a maximum of 2 mmol/l in a few NAFLD patients and in diabetic subjects.

Insulin levels were tested every 15 min in the last hour of the clamp, whereas free fatty acids (FFAs) were measured at the beginning and end of the insulin infusion period.

Measurement of hepatic glucose production. In 10 patients with NAFLD (5 patients with type 2 diabetes and 5 control subjects) hepatic glucose production (HGP) was measured using [6,6-$^2H_2$]glucose in the basal state and during the clamp. To do so, a 2-h basal period was added to the study protocol; subjects received a primed (2.5 mg/kg) continuous (2.0 mg · kg^{-1} · h^{-1}) infusion of the tracer in the 2 h before the clamp and throughout the period of insulin infusion. Isotope was also added to the 20% dextrose solution used to maintain euglycemia in the course of the clamp in an amount that yielded an isotopic enrichment approximately equal to that in plasma at isotopic equilibrium (27). Three plasma samples were obtained at 5-min intervals before isotope infusion, the last 10 min of the 2-h equilibration period, and during the last 10 min of the insulin clamp. HGP was calculated by subtracting the

TABLE 2
Clinical features of the metabolic syndrome in NAFLD patients

	Prevalence in percent (95% CI)
Obesity	
Overweight (BMI 25.0–29.9 kg/m^2)	67 (47–83)
Central fat accumulation	
Waist circumference >102 cm (men) or 88 cm (women)	47 (28–66)
Impaired glucose regulation	
Increased fasting insulin (>100 pmol/l)	57 (38–75)
Postload hyperinsulinemia (>1,000 pmol/l)	27 (12–46)
Family history of diabetes (first-degree relatives)	47 (28–66)
Hypertriglyceridemia (>2 mmol/l)	47 (28–66)
Hyperuricemia (>400 μmol/l)	27 (12–46)
Low HDL cholesterol (<1 μmol/l)	43 (25–63)
Hypertension (>160/95 mmHg or actively treated)	17 (6–35)
Family history of hypertension (first-degree relatives)	57 (38–75)
Cardiovascular disease (previous acute myocardial infarction or angina)	0 (0–12)
Family history of cardiovascular disease (first-degree relatives)*	27 (12–46)

*Male relatives <55 years of age, and female relatives <60 years of age.

exogenous glucose infusion rate (20% glucose infusion to maintain euglycemia during the clamp) from the total rate of isotope appearance in serum.

Laboratory methods. Plasma glucose, both in the fasting state and in response to a standard glucose load, was measured in duplicate with an automated analyzer. The coefficient of variation for any single determination was ± 1.5%. Insulin was measured by an immunoenzymometric assay (AIA-PACK IRI; AIA-1200 System; Tosoh, Tokyo, Japan) with intra- and interassay coefficients of variation <7% for quality control. C-peptide was measured by radioimmunoassay (Liso-phase; Tecnogenetics, Milan, Italy), with coefficients of variation <13%. The average insulin concentration during OGTT was calculated by means of the trapezoidal rule. Fasting serum cholesterol, HDL cholesterol, uric acid levels, and triglyceride levels were measured by routine laboratory techniques. FFAs were measured by an enzymatic colorimetric method (NEFA C; Wako Chemicals, Neuss, Germany).

Plasma samples were derivatized for the measurement of plasma [6,6-$^2H_2$] glucose enrichment by gas chromatography/mass spectrometry (Metabolic Solutions, Boston, MA). Results were reported as moles of percent excess.

Statistical analyses. All analyses were carried out on a personal computer with the StatView 5.0 program (SAS Institute, Cary, NC.). Results were expressed as means ± SD for each subject group and each variable. The significance of difference between groups was tested using analysis of variance (ANOVA) and unpaired Student's *t* test. Metabolic parameters were also tested for differences between groups by analysis of covariance, after adjustment for age, BMI, and waist-to-hip ratio. Correlation between variables was tested by means of parametric and nonparametric (Spearman rank correlation) methods. Several sets of variables were simultaneously tested (i.e., anthropometric measurements, fasting and postload glucose and insulin, glucose disposal and insulin during the clamp, HGP, basal and insulin-suppressed FFA concentrations, iron status, and lipid profile). Accordingly, the limit of significance was adjusted following Duncan's multiple range (28) to

$$P' = 1 - \sqrt[(n-1)]{(1 - P)}$$

where $P = 0.05$ and $n = 9$. The final critical value of significance was therefore set at 0.005.

RESULTS

Clinical and laboratory data. When compared with control subjects, NAFLD patients were only moderately overweight but had a larger waist circumference. Fasting and postload glucose values were normal or high-normal, whereas fasting insulin was increased threefold ($P = 0.0001$). Their BMI was slightly lower than in the diabetic patients, whereas their average waist circumference and waist-to-hip ratio was larger. The lipid profile showed a mild hypercholesterolemia, reduced HDL cholesterol concentrations, and higher-than-normal triglyceride and uric acid levels (Table 1). Most of these abnormalities were of the same order of magnitude as the corresponding values measured in the diabetic patients. One or more features of the metabolic syndrome were present in all NAFLD patients, variably associated with each other (Table 2).

TABLE 3
Histologic scoring of NAFLD patients

Grading	Fat infiltration	Fibrosis	Necroinflammation
Grade 0	—	17 (6–35)	30 (15–49)
Grade 1	50 (31–69)	70 (51–85)	53 (34–72)
Grade 2	23 (10–42)	13 (4–31)	13 (4–31)
Grade 3	27 (12–46)	0 (0–12)	3 (0–17)

Data are % cases (95% CI). Grading performed according to Diehl et al. (12).

Transaminase levels were increased to a maximum of more than five times the normal values. ALTs exceeded aspartate transaminases (ASTs) in all cases, the latter being normal in 53% of cases (95% CI 34–72).

Of the 30 patients, 21 (80%; 95% CI 61–92) had a liver biopsy compatible with fatty liver associated with necroinflammation and fibrosis and could be classified as NASH, whereas 9 patients had no inflammation or fibrosis (pure fatty liver). The extent of fat deposition, fibrosis, and necroinflammation was variable (Table 3), but no subjects had evidence of cirrhosis. There was no correlation between fat and necroinflammation ($r_s = 0.182$, $P = 0.328$; Spearman's rank correlation).

Iron status was suggestive of iron overload in 10 or 43% of cases, depending on the use of serum iron or ferritin as markers (Table 4). Only 4 patients were heterozygous for mutation His63Asp of the HFE gene, whereas no mutation Cys282Tyr was demonstrated. There was no correlation among serum iron, HFE gene mutation, and clinical or laboratory features (not reported in detail).

When patients were stratified according to the presence of pure fatty liver or NASH, no differences were observed in clinical and metabolic parameters, including transaminase levels (ALT 68 ± 23 vs. 100 ± 42 units/l and AST 36 ± 8 vs. 44 ± 17 units/l for pure fatty liver and NASH, respectively). However, although ALT levels did not correlate with fat ($r_s = 0.419$; P corrected for ties = 0.024; Spear-

TABLE 4
Iron status in NAFLD patients

	Mean ± SD (range)	Cutoff value	Prevalence in percent (95% CI)
Serum iron (μmol/l)	18 ± 5 (9–32)	>25	10 (2–27)
Total transferrin (μmol/l)	61 ± 14 (33–118)	<45	7 (1–22)
Transferrin saturation (%)	30 ± 8 (14–50)	>33	30 (15–49)
Ferritin (ng/ml)	301 ± 304 (22–1581)	>250	43 (25–63)
Heterozygosity for HFE	4 cases*	—	13 (4–31)

Prevalence given is of values outside normal range. *All cases had a heterozygosity for mutation His63Asp.

TABLE 5
Euglycemic clamp data in the three study groups

	NAFLD patients	Type 2 diabetic patients	Control subjects
n	30	10	10
Steady-state glucose (mmol/l)	5.0 ± 0.3 (4.4–5.6)	5.1 ± 0.2 (4.9–5.5)	4.9 ± 0.2 (4.6–5.2)
Steady-state insulin (pmol/l)	667 ± 103 (487–940)	722 ± 122 (581–918)*	590 ± 55 (531–696)
Glucose infusion rate (μmol · kg^{-1} · min^{-1})	19.5 ± 5.7 (10.4–33.6)*	16.2 ± 4.0 (10.8–23.2)*	37.2 ± 9.4 (24.7–58.4)
Glucose disposal rate (μmol · kg^{-1} · min^{-1})	18.4 ± 5.9 (8.2–31.6)*	18.0 ± 4.0 (13.2–26.1)*	36.8 ± 7.6 (26.2–54.7)
Fasting FFAs (mmol/l)	0.67 ± 0.27 (0.26–1.30)*	0.55 ± 0.11 (0.44–0.81)*	0.37 ± 0.12 (0.23–0.61)
End-of-clamp FFAs (mmol/l)	0.21 ± 0.14 (0.05–0.55)*	0.13 ± 0.05 (0.06–0.20)*	0.06 ± 0.02 (0.03–0.10)

Data are means ± SD (range). *$P < 0.001$ vs. control subjects.

man's rank correlation) or with fibrosis ($r_s = 0.050$; $P = 0.788$), there was an association of ALT with necroinflammation ($r_s = 0.622$; $P = 0.0008$).

Clamp study. Continuous insulin infusion resulted in steady-state insulin concentrations in the 450–900 pmol/l range in the different experiments (Table 5). In the diabetic patients, steady-state insulin levels were 20% higher ($P = 0.006$) than in control subjects. Glucose concentrations in the last 40 min of insulin infusion were similar among groups and varied by <5% in all experiments. The amount of glucose infused to maintain euglycemia was reduced by nearly 50% in NAFLD patients, an extent similar to that observed in diabetic patients (Table 5 and Fig. 1). Differences in glucose disposal between NAFLD patients and control subjects did not change after adjustment for age ($P = 0.286$), BMI ($P = 0.192$), and waist-to-hip ratio ($P = 0.586$).

Plasma FFAs were moderately increased in NAFLD patients in the fasting, preinfusion state (Table 5 and Fig. 1). In response to insulin, they decreased in all groups, but less efficiently in NAFLD patients compared with control subjects ($P = 0.003$; percent of basal values: NAFLD patients, −69 ± 14%, range 42–93; control subjects, −84 ± 5%, range 74–92; type 2 diabetic patients, −76 ± 8, range 63–91). Fasting and insulin-suppressed FFA levels did not correlate with the histological degree of hepatic fat infiltration ($r_s = 0.028$ and 0.062, respectively; Spearman's rank correlation).

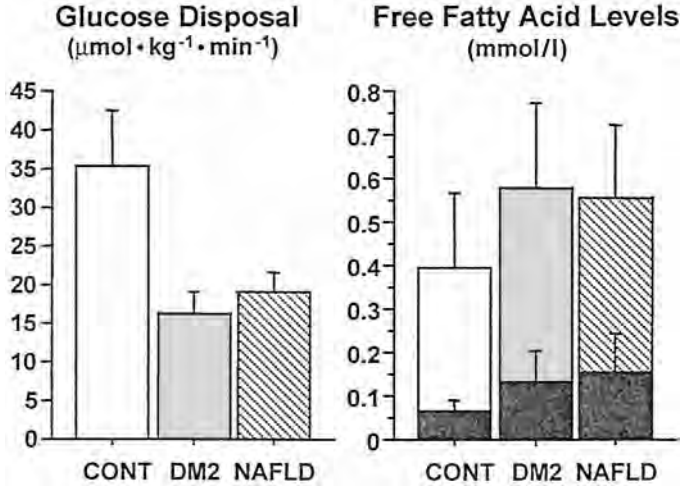

FIG. 1. Glucose disposal in the course of the clamp and FFA concentrations in control subjects (CONT; open column; $n = 10$), patients with type 2 diabetes (DM2; shaded column; $n = 10$), and NAFLD subjects (hatched column; $n = 30$). Black bars represent FFA levels at the end of the clamp study. Data are presented as means and 95% CI.

Glucose disposal significantly correlated with BMI ($r = -0.490$; $P < 0.001$) and waist circumference ($r = -0.492$; $P < 0.001$) when all experiments were pooled ($n = 50$) (Fig. 2). When the analysis was limited to NAFLD patients, no significant correlations were found (BMI, $r = -0.041$; waist circumference, $r = 0.187$).

In NAFLD patients, there were no differences in glucose disposal in relation to the absence/presence of overweightness (for BMI <25 kg/m^2: 19.1 ± 3.9 μmol · kg^{-1} · min^{-1}; for BMI ≥25, 18.1 ± 6.7 μmol · kg^{-1} · min^{-1}; $P = 0.654$). Similarly, glucose disposal did not correlate with serum iron ($r = 0.265$) or serum ferritin concentrations ($r = -0.177$) and was not different in patients with pure fatty liver compared with NASH patients (22.2 ± 5.3 vs. 16.8 ± 5.5 μmol · kg^{-1} · min^{-1}, respectively; $P = 0.02$).

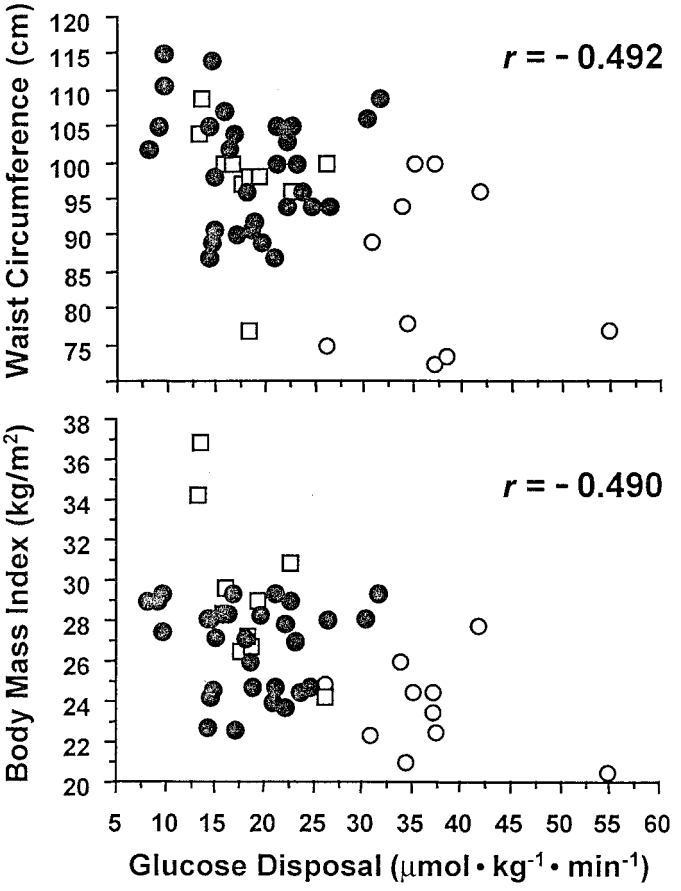

FIG. 2. Correlation between glucose disposal, measured in the course of the euglycemic clamp, and waist circumference and BMI in the whole population under study. ○, control subjects; ●, NAFLD patients; □, type 2 diabetic patients.

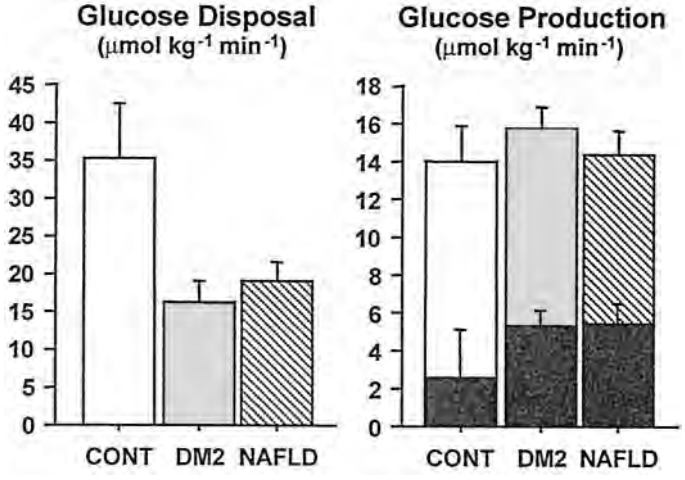

FIG. 3. Glucose disposal in the course of the clamp and hepatic glucose production in the subgroup of subjects infused with [6,6-$^2H_2$]glucose. The subgroups—control subjects (CONT; open columns; $n = 5$), type 2 diabetic patients (DM2; shaded columns; $n = 5$), and NAFLD subjects (hatched columns; $n = 10$)—are representative of the whole population. Black bars represent hepatic glucose production at the end of the clamp study. Data are presented as means and 95% CI.

Hepatic glucose output. The subgroups of patients whose hepatic glucose output was measured by deuterated glucose were fully representative of the three populations under study, in terms of anthropometry and liver disease (not reported in detail) as well as glucose and lipid regulation and insulin sensitivity. In particular, fasting glucose was 5.0 ± 0.27 vs. 6.4 ± 0.45 vs. 5.4 ± 0.55 mmol/l; FFAs were 0.39 ± 0.14 vs. 0.58 ± 0.15 vs. 0.56 ± 0.23 mmol/l; and glucose disposal during the clamp was 35.3 ± 5.7 vs. 16.3 ± 2.3 vs. 19.0 ± 3.4 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ in control subjects, diabetic patients, and NAFLD patients, respectively (Fig. 3).

Basal HGP (Fig. 3) was normal in NAFLD patients compared with control subjects (14. 3 ± 3.4 vs. 14.0 ± 2.4 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, respectively; NS) and only moderately increased in diabetic patients (15.7 ± 0.8 $\mu mol \cdot kg^{-1} \cdot min^{-1}$; $P = 0.06$ vs. control subjects). In response to insulin infusion, HGP decreased to a final value of −63 ± 9% of basal in NAFLD patients (5.4 ± 2.6 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) compared with −82 ± 14% of basal in control subjects (2.6 ± 4.4 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) (Fig. 3). The difference in percent suppression was significant at $P = 0.002$. Compared with diabetic patients, NAFLD was characterized by a similar HGP in the basal state and a similarly impaired insulin suppression of glucose output during the clamp (−65 ± 4% in diabetic patients).

DISCUSSION

Our study confirmed that NAFLD is characterized by a remarkable reduction of insulin sensitivity, with decreased insulin effects on both glucose and lipid metabolism. Such a defect is not exclusively associated with the abnormal glucose regulation and/or overweightness, as is frequently observed in NAFLD, but also extends to subjects with normal weight and normal glucose tolerance. This suggests that insulin resistance might be a primary phenomenon, sometimes adding to obesity- and diabetes-associated insulin resistance. This conclusion is based on the measurement of glucose disposal during the euglycemic-hyperinsulinemic clamp; in the presence of steady-state insulin concentrations moderately increased in comparison to control subjects and similar to that observed in type 2 diabetic subjects, glucose disposal was nearly halved in a series of biopsy-proven NAFLD patients.

To rule out diabetes as a primary cause of insulin resistance, only NAFLD subjects with normal fasting glucose and glucose tolerance were selected for the present study. We also ruled out the possibility that obesity played a role; although total fat mass and intra-abdominal fat were not quantitatively measured, 33% of patients had a normal body weight. Recalculating glucose disposal per kilogram of lean body mass (LBM), predicted by the dated Hume formula (29), does not quantitatively change the results (50.4 ± 9.1 vs. 27.5 ± 4.8 vs. 27.0 ± 8.8 $\mu mol \cdot kg^{-1}$ LBM $\cdot$ min^{-1} in control subjects, diabetic patients, and NAFLD patients, respectively, by ANOVA; $P < 0.0001$). This quantitative analysis was confirmed by the lack of difference in glucose disposal between normal- and overweight NAFLD subjects and the lack of correlation between BMI and glucose disposal in the NAFLD series. Our data are supported by the recent finding that fasting hyperinsulinemia and reduced glucose tolerance, markers of insulin resistance, are present in both obese and normal-weight NAFLD patients (30), although the total amount of hepatic fat deposition seems to be proportional to the degree of obesity (6).

NAFLD, irrespective of obesity and fasting glucose levels, might be included in the metabolic syndrome (31), having obesity and type 2 diabetes as main clinical features (14), but also associated with raised triglyceride levels, hypertension (32), and splanchnic fat distribution (33). Previous studies have associated liver disease and raised transaminases to the metabolic syndrome only in severe obesity (34), suggesting that NAFLD might be a secondary phenomenon. In the present study, the association of fatty liver with features of the metabolic syndrome was independent of BMI. Most patients, including lean patients, had a family history of diabetes or hypertension, fitting the proposed criteria for the insulin-resistance syndrome (31) and pointing to a genetic defect, sometimes magnified by other phenotypic expressions.

By using the glucose clamp technique, we intended to estimate insulin sensitivity quantitatively and validate previous results obtained by HOMA calculation (17). The HOMA measure, which considers only glucose and insulin concentrations under fasting, nondynamic conditions, is expected to give a picture of basal insulin effects, mainly on the liver, considering the importance of HGP on fasting hyperglycemia (35). The euglycemic clamp, measuring glucose disposal under hyperinsulinemia and unlimited substrate availability, gives a broader evaluation of insulin sensitivity and, when coupled with tracer methodology, more clearly defines the site of insulin resistance.

We showed that in NAFLD patients, HGP is normal or high-normal in the fasting state, despite basal hyperinsulinemia, and that the liver does not switch off glucose production in response to insulin infusion. Therefore, both during fasting and in response to glucose ingestion, the liver itself might be responsible for insulin resistance. Comparing NAFLD with type 2 diabetes in our patients, it appears that the two conditions are very similar in the

presence of different fasting glucose and overall glucose metabolism.

Insulin resistance in NAFLD might theoretically derive from liver damage itself, as repeatedly demonstrated in patients with cirrhosis (36). In the current study, this hypothesis was ruled out by the normal liver function in all our patients, with the exception of the raised transaminase values. Only the small difference in glucose disposal between NAFLD patients and subjects with pure fatty livers might be attributable to additional liver disease that is undetectable either clinically or in laboratory tests.

Decreased insulin sensitivity also involves lipid metabolism. Hypertriglyceridemia was shown to be associated with NAFLD (17), together with increased FFA levels, which were nearly doubled in the present study. FFAs are responsible for reduced insulin clearance (37)—hence the moderate hyperinsulinemia observed during the clamp in NAFLD as well as in type 2 diabetic patients (38).

In the present study, FFA levels were not systematically associated with overweightness or central fat accumulation, which are involved in lipid deposition in the hepatic parenchyma (38,39) via increased lipolysis and high visceral fat turnover. In addition, there was no correlation between the degree of hepatic steatosis, documented by liver biopsy, and FFA levels. A striking lipid abnormality in NAFLD patients was the lower-than-normal decrease of plasma FFAs in response to insulin infusion, which suggested a decreased insulin-mediated suppression of lipolysis. Whether this defect is the cause or an effect of hepatic lipid deposition remains to be determined. Further studies would also clarify the role of FFA metabolism on decreased insulin sensitivity.

The possibility remains that hepatic fat deposition derives from the combined effects of an unknown, genetic combination responsible for reduced insulin sensitivity and acquired conditions (namely, central obesity and increased visceral fat turnover) (40). The role of central obesity deserves special attention. A previous study linking NAFLD to insulin resistance in normal weight subjects was carried out in Koreans (30) (Asians have been observed to have increased visceral adiposity in the presence of normal body weight). In the present series, the waist circumference and waist-to-hip ratio of NAFLD subjects were moderately larger than those of the diabetic patients, despite similar BMIs. In the NAFLD group, subjects with normal body weight had a waist-to-hip ratio of the same order of magnitude as overweight subjects (0.93 ± 0.07 vs. 0.95 ± 0.06, respectively; $P = 0.429$) and larger than control subjects (0.83 ± 0.09; $P = 0.02$). In summary, NAFLD patients, even in the presence of normal body weight, have a relatively increased visceral adiposity, which might be relevant in the pathogenesis of their liver disease.

Whatever the cause of hepatic lipid deposition, it may remain clinically undetectable or may cause disorders from ongoing liver damage to terminal liver failure needing hepatic transplantation. To explain differences between pure fatty liver and NASH, Day and James (41) proposed a "two-hit hypothesis," with lipids acting as the first "hit" and increased lipid peroxidation being the second "hit." To explain oxidative stress, another culprit may be present but not necessarily the same agent in all cases. In the absence of potentially hepatotoxic drugs, genetic conditions such as hemochromatosis (42), dietary habit (43), as well as acquired deficiencies in antioxidant systems (mainly vitamins) (44) may be involved.

The role of iron deposition in the pathogenesis of NAFLD has raised general interest (45). Moirand et al. (46) first associated primary hepatic iron overload with the clinical features of insulin resistance, irrespective of liver damage. Most patients with primary hepatic iron overload fit the criteria of NAFLD (47). Our study confirmed that serum indexes of iron overload (increased ferritin, low unsaturated transferrin, and higher-than-normal transferrin saturation) are present in most patients with NAFLD and that a nonnegligible proportion of these patients is heterozygous for mutation His63Asp of the hemochromatosis gene HFE.

The mechanism responsible for liver damage in hepatic iron overload probably involves several steps. Hepatic iron can directly cause lipid peroxidation (48), and a product of lipid peroxidation (namely, malonyldialdehyde) has been shown to activate stellate cells (49) and increase collagen production (50). However, many NAFLD patients show no evidence of hepatic iron overload, and no differences are present in clinical features in relation to iron status (42). More importantly, iron status does not classify patients according to the histological severity of their liver disease (13). We also demonstrated that serum indexes of iron overload do not correlate with measures of insulin sensitivity. Accordingly, hepatic iron might be one but not the only agent causing ongoing fatty liver disease. Any drug-induced (51) or occupational damage (52) leading to lipid peroxidation might be responsible, but in the majority of cases this issue is not settled; in addition, the degree of fat infiltration does not correlate with necroinflammation. A role of cytokines, namely tumor necrosis factor-α, secreted in response to steatosis-induced lipid peroxidation, has also been suggested (53).

The possible relevance of impaired insulin sensitivity in the pathogenesis of NAFLD has potential therapeutic implications that need to be tested in clinical studies. In overweight or obese subjects, a weight-reducing nutritional regimen is likely to reduce the obesity-related insulin resistance, which might be partly responsible for liver fat deposition (7). In all patients, using metformin or glitazones to increase hepatic sensitivity to insulin might break the vicious circle linking hyperinsulinemia and insulin resistance to elevated triglyceride and FFA concentrations, progressive steatosis, ultrastructural mitochondrial lesions in the hepatocytes, and ultimately hepatocyte death (54). Such a hypothesis has been recently tested in an animal model (55). Data in NAFLD patients are eagerly awaited.

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