

CLINICAL STUDY

Plasma adiponectin is decreased in nonalcoholic fatty liver disease

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Abstract

Objectives: Nonalcoholic fatty liver disease (NAFLD) is a major cause of liver-related morbidity and is frequently associated with obesity and metabolic syndrome. The recently discovered hormone adiponectin is produced by adipose tissue, and low plasma adiponectin is considered a key factor in the development of the insulin resistance underlying metabolic syndrome. Animal studies suggest that adiponectin may protect against non-alcoholic steatohepatitis, but direct evidence in humans is lacking. We therefore conducted this study to assess the relationship between plasma adiponectin and nonalcoholic fatty liver disease to explore its role in the pathogenesis of this disease.

Design and methods: We measured plasma adiponectin and anthropometric, biochemical, hormonal and metabolic correlates in a group of 17 NAFLD patients with diagnosis confirmed by biopsy, and 20 controls with comparable age, body-mass index and sex. Furthermore we compared plasma adiponectin in patients with simple steatosis and steatohepatitis.

Results: Plasma adiponectin was significantly lower in NAFLD patients than controls (5.93 ± 0.45 vs 15.67 ± 1.60 ng/ml). Moreover, NAFLD patients were significantly more insulin resistant while having similar serum leptin. Adiponectin was similar in simple steatosis and in steatohepatitis (6.16 ± 0.78 vs 5.69 ± 0.49 ng/ml). An inverse correlation was observed between adiponectin and homeostatic model assessment (HOMA) of insulin resistance ($P = 0.008$), while adiponectin did not correlate with serum transaminases and lipid values.

Conclusions: These data support a role for low circulating adiponectin in the pathogenesis of NAFLD and confirm the strict association between reduced adiponectin production by adipose tissue, NAFLD and insulin resistance.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of disorders characterized by macrovesicular hepatic steatosis occurring in individuals without a relevant alcohol consumption. It is a complex metabolic condition in which both lifestyle and genetic factors have a pathogenic role (1) and has been increasingly recognized as a major cause of liver-related morbidity and mortality (2). Moreover, NAFLD has been convincingly associated with the metabolic insulin-resistance syndrome; most patients are overweight or frankly obese, with altered glucose regulation, dyslipidemia, and raised blood pressure, all contributing to the disorder (3). However, large studies have shown that approximately 10–20% of patients are lean and have normal glucose regulation, but are nonetheless insulin resistant when tested by the homeostatic

model assessment (HOMA) of insulin resistance method (4) or by the euglycemic clamp technique (5). Insulin resistance, through the inhibition of lipid oxidation and increased fatty acid and triglycerides synthesis, is believed to be a key factor in the development of fatty liver. Moreover, insulin resistance states, such as obesity and diabetes, are also characterized by elevated expression and production of several cytokines; of particular interest are cytokines produced by adipose tissue, the so-called adipokines (adiponectin, leptin, resistin, TNF α , TGF β and PAI-1). Their role in the development of diabetes and other obesity complication has been suggested (6). Adiponectin, also known as ACRP30, is a 30 kDa protein abundantly and selectively expressed in white adipose tissue. Its role in insulin resistance and atherosclerosis has been well established. Recently, two adiponectin receptors have been cloned in mouse and humans, and both

are expressed in liver (7). Although the available evidence indicates that adiponectin stimulates fatty acid oxidation in liver and skeletal muscle, it is still unknown whether circulating adiponectin levels are altered in disorders of hepatic metabolism of energy substrates. Current data suggest that adiponectin may have a protective role in liver injury in alcoholic and nonalcoholic fatty liver in mice (8), but direct evidence of the role of adiponectin in human NAFLD is lacking. Therefore, the aim of this study was to determine circulating adiponectin levels in patients affected by NAFLD and to correlate plasma adiponectin with insulin sensitivity and anthropometric variables, liver function and histologic feature.

Patients and methods

Seventeen NAFLD patients (15 males, 2 females) and 20 control subjects (17 males, 3 females), matched for age, sex and body-mass index (BMI), were included in the study. Their clinical and laboratory parameters are illustrated in Table 1. NAFLD diagnosis was based on chronic elevation of transaminases (>1.5 times the upper normal value for 3 months or longer), absence of hepatitis B and C virus markers, absence of autoantibodies indicative of autoimmune hepatitis or celiac disease, absent or negligible alcohol consumption (<20 g/day), and bright liver at ultrasound scanning. In all patients, diagnosis was confirmed by liver biopsy. NAFLD patients were subsequently divided into pure fatty liver and nonalcoholic steatohepatitis (NASH). Eight cases were classified as NASH, and nine were classified as pure fatty liver by the criteria proposed by Brunt *et al.* (9). The control group was age, weight and sex matched and was free from hepatic, neoplastic and endocrine diseases. NAFLD was excluded by normal transaminase values and normal liver ultrasound. Subjects with hypertension were also excluded. Previously diagnosed diabetic patients, according to the American Diabetes Association classification, were excluded by both the study and the control group. None of the NAFLD patients and control subjects were taking lipid-lowering medications, metformin or thiazolidinediones. All patients were recruited in the outpatient clinic and were on an unrestricted dietary regimen. Body weight was stable

Table 1 Anthropometric and clinical characteristics of NAFLD patients and control subjects. Data are mean \pm S.E.M. Statistical analysis performed by ANOVA.

	NAFLD	Control	P value
M/F	15/2	17/3	NS
Age (years)	44 \pm 3	42 \pm 4	NS
Body weight (kg)	86.2 \pm 3.5	77.5 \pm 4.0	NS
BMI (kg/m ²)	27.4 \pm 0.8	26.6 \pm 1.0	NS
Height (m)	1.77 \pm 0.02	1.70 \pm 0.03	NS

during the 3 months preceding the study. A blood sample for determination of adiponectin, glucose, insulin, alanine aminotransferase (ALT), γ -glutamyl-transpeptidase (γ GT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, triglycerides and leptin concentration was collected at 0800–0900 h, after overnight fasting. Plasma was immediately separated and frozen and stored at -80°C until analysis. Written informed consent was obtained from all patients for blood sampling for adiponectin and leptin measurement, and for the anonymous use of their clinical data after explanation of the purpose of the study. All other investigations were carried out as standard procedures for diagnosis and follow-up of NAFLD.

Analytic procedures

AST, ALT, GGT, ALP, total, HDL and LDL cholesterol, and triglycerides were measured by automated enzymatic methods. Glucose was measured in triplicate by the glucose oxidase method (Beckman Glucose Analyzer, Beckman, Palo Alto, CA, USA). Insulin and leptin were measured by commercially available radioimmunoassay kits (Linco Research, St. Charles, MO, USA). Adiponectin was measured by radioimmunoassay with an antibody against human adiponectin and dilution of recombinant adiponectin as standard. Inter- and intra-assay coefficients of variation were $<10\%$ and $<5\%$ respectively (Linco Research, St. Charles, MO, USA).

Statistical analysis

Results are expressed as mean \pm S.E. Different groups were compared by Student's *t*-test for independent values with Bonferroni correction. Correlations between different variables were determined by logarithmic regression and multivariate analysis with the Statistica software package (StatSoft, Tulsa, OK, USA). *P* values less than 0.05 were considered significant.

Results

Anthropometric, clinical and biochemical parameters are illustrated in Tables 1 and 2. No significant differences were observed in fasting glucose while both plasma insulin and HOMA were significantly higher in NAFLD patients than control subjects. Total cholesterol, LDL cholesterol and triglycerides were all slightly, but not significantly, higher in NAFLD patients. Serum leptin was similar in NAFLD and control group. Plasma adiponectin was significantly lower in NAFLD patients than controls, while no difference was observed between patients with steatohepatitis and pure fatty liver (Fig. 1). When correlation analysis was performed, adiponectin levels were inversely correlated with fasting insulin ($r = 0.40$; $P < 0.05$) and with HOMA

Table 2 Biochemical parameters of NAFLD and control subjects. *P* values refer to comparison between control and NAFLD group. NAFLD patients were also divided into two subgroups representing patients with pure fatty liver and steatohepatitis. However, no significant differences were found between these subgroups. Reference value of HOMA in a normal-weight, nondiabetic population from our database is 1.45 ± 0.21 (mean \pm s.e.m.).

	NAFLD (n = 17)	Pure fatty liver (n = 9)	Steatohepatitis (n = 8)	Control (n = 20)	<i>P</i> (Control vs NAFLD)
Fasting glucose (mmol/l)	5.95 \pm 0.42	5.99 \pm 0.50	5.88 \pm 0.48	5.33 \pm 0.26	NS
Fasting insulin (mU/l)	13.4 \pm 1.5	13.9 \pm 2.0	12.7 \pm 2.2	8.7 \pm 0.6	0.006
Fasting lipids					
Total cholesterol (mmol/l)	5.41 \pm 0.31	5.50 \pm 0.50	5.19 \pm 0.20	4.90 \pm 0.59	NS
HDL cholesterol (mmol/l)	1.24 \pm 0.10	1.15 \pm 0.20	1.29 \pm 0.28	1.50 \pm 0.19	NS
LDL cholesterol (mmol/l)	3.24 \pm 0.29	3.12 \pm 0.30	3.29 \pm 0.31	2.78 \pm 0.45	NS
Triglycerides (mmol/l)	2.04 \pm 0.35	2.15 \pm 0.45	2.00 \pm 0.30	1.71 \pm 0.55	NS
HOMA	3.61 \pm 0.55	3.30 \pm 0.40	3.75 \pm 0.60	1.95 \pm 0.26	0.01
AST (U/l)	35 \pm 3	34 \pm 4	35 \pm 2	20 \pm 1	<0.001
ALT (U/l)	68 \pm 8	71 \pm 14	64 \pm 7	21 \pm 1	<0.001
γ GT (U/l)	94 \pm 26	99 \pm 38	84 \pm 33	17 \pm 4	<0.001
ALP (U/l)	97 \pm 14	114 \pm 36	89 \pm 10	65 \pm 16	NS
Leptin (ng/ml)	9.13 \pm 1.15	9.40 \pm 1.30	8.80 \pm 1.70	9.89 \pm 1.18	NS

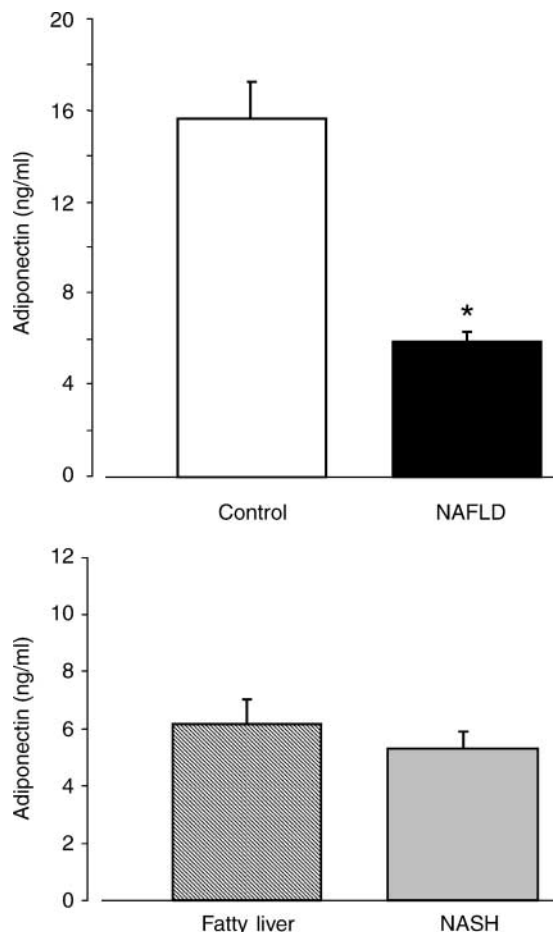


Figure 1 Plasma adiponectin in patients with NAFLD and control subjects (upper panel) and in NAFLD patients divided according to histologic diagnosis (pure fatty liver vs steatohepatitis) (lower panel). Data are expressed as mean \pm s.e.m. **P* < 0.001 vs control.

($r = 0.45$; $P = 0.008$) (Fig. 2). No significant correlation was found between adiponectin and BMI, fasting glucose, leptin and liver function tests (AST, ALT, γ GT and ALP). On multiple regression analysis, adiponectin maintained its significant correlation with HOMA ($B = -2.74$; $P = 0.01$) when age and BMI were included in the statistical model.

Discussion

This study essentially shows that NAFLD patients have reduced circulating adiponectin and this reduction is associated with insulin resistance. The strong association between insulin resistance and NAFLD has been extensively demonstrated in recent years, and several authors have proposed to include NAFLD in the complex picture of the metabolic syndrome (3). Available evidence suggests that insulin resistance affects hepatic fat accumulation by increasing release of free fatty acids from adipose tissue, increasing fatty acid and triglycerides synthesis in the liver, reducing fatty acid oxidation and reducing very low-density lipoprotein (VLDL) production. Insulin resistance and hyperinsulinemia are also associated with the inflammatory and fibrotic reaction that complicates advanced stages of the disease (10). Moreover, obesity and insulin resistance have been found to worsen the evolution of hepatitis C virus (HCV)-related liver steatosis by independently promoting or increasing liver steatosis (11, 12).

However, the discovery of adiponectin has added an additional potential mechanism to explain the pathogenesis of liver steatosis. Adiponectin produces relevant effects on the hepatic metabolism of energy substrates. Experimental studies in rodents showed that infusion of recombinant adiponectin inhibits basal endogenous glucose production (EGP) and potentiates insulin-induced inhibition of EGP (13). This is due to lowered gluconeogenesis and to altered expression and activity

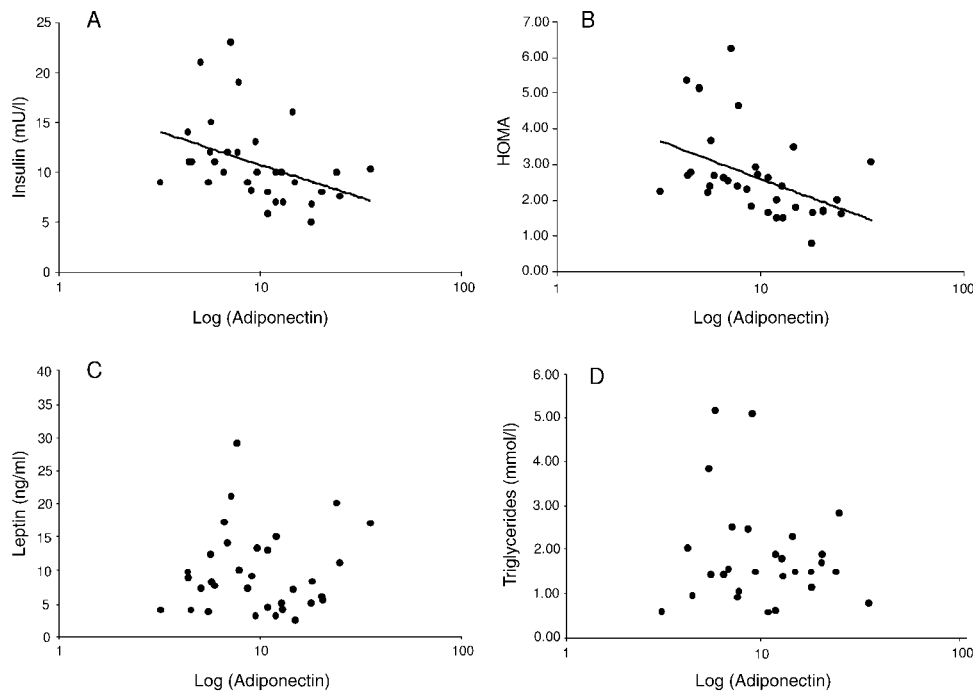


Figure 2 Regression analysis between plasma adiponectin and insulin (A), HOMA (B), leptin (C) and triglycerides (D) in NAFLD patients and in control subjects. Significant correlations were found between adiponectin and insulin ($y = -2.8721\ln(x) + 17.38$; $r = 0.40$, $P < 0.02$) and between adiponectin and HOMA ($y = -0.923\ln(x) + 4.72$; $r = 0.45$, $P = 0.008$).

of key enzymes of gluconeogenesis (8). Furthermore, adiponectin stimulates fatty acid oxidation in hepatocytes.

The effect of adiponectin on liver metabolism was confirmed *in vivo* in humans by the strong association between plasma adiponectin and both basal and 2-h clamp EGP (14). Interestingly, this association is independent of peripheral insulin sensitivity, thus supporting the hypothesis that liver is a major target of adiponectin. Recently, two types of adiponectin receptors (AdipoR1 and AdipoR2) have been cloned, and both types are expressed in the liver. Binding of adiponectin to its receptors stimulates phosphorylation of AMPK, PPAR α activity and fatty acid oxidation in liver (7), and this mechanism is inhibited by resistin. Moreover, liver AdipoR1 and AdipoR2 are rapidly downregulated by insulin and in *ob/ob* mice, thus suggesting that not only hypoadiponectinemia, but also 'adiponectin resistance' may be involved in disorders of liver metabolism (15, T Kadowaki, personal communication).

Moreover, it was recently reported that also in humans expression of adiponectin receptor in skeletal muscle is directly correlated with insulin sensitivity (16). The low adiponectin levels in NAFLD patients reported in this study may represent a pathogenic mechanism leading to altered hepatocyte lipid metabolism and fat accumulation. In fact, high adiponectin levels have been reported to protect against both alcoholic and nonalcoholic fatty liver disease in mice by reducing fatty acid synthesis through inhibition of acyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) expression and activity (8). The reduction of

ACC activity reduces the malonyl-CoA level, which is known to inhibit carnitine palmitoyltransferase I (CPT-I) activity and fatty acid oxidation. Therefore, reduced adiponectin in NAFLD could result in increased fatty acid synthesis, accumulation of triglycerides, and reduced fatty acid oxidation.

However, adiponectin may protect against steatohepatitis also through its anti-inflammatory action. It is well known that inflammation is a key mechanism in the progression of fatty liver to hepatitis and cirrhosis (17). Adiponectin inhibits liver TNF α expression (8, 18) and also inhibits expression of several cytokines in hepatic stellate cells (HSC) (19). Moreover, adiponectin reduces lipid accumulation and inflammation in alcohol-induced liver injury (8). We did not find any difference in circulating adiponectin between patients with histologic diagnosis of pure steatosis and those with steatohepatitis. However, it is not possible to exclude that peripheral adiponectin concentration does not reflect adiponectin concentration in the portal vein, or that differences in expression of adiponectin receptor may be relevant to the progression of fatty liver to NASH and to fibrosis.

Another adipokine supposed to have a crucial role in liver fibrosis is leptin. In fact, leptin increases TNF α , TGF- β 1 and type 1 collagen expression in liver, while *fa/fa* Zucker rats, which are resistant to leptin due to a leptin receptor defect, are resistant to thioacetamide-induced liver fibrosis (20). However, a direct association between leptin and NAFLD was not confirmed *in vivo* in humans by Chalasani *et al.* (21), who found no correlation between circulating leptin and leptin receptor expression in liver biopsies and

nonalcoholic fatty liver patients. The lack of difference in plasma leptin between NAFLD patients and controls in our study does not further support a role for leptin in the development of NAFLD.

The relationship between adiponectin and NAFLD is supported by a recent report by Bajaj *et al.*, who found an inverse correlation between adiponectin levels and liver fat content in diabetic patients as measured by nuclear magnetic resonance (22). Recently, an association between plasma adiponectin and liver function was found also in healthy subjects. López-Bermejo *et al.* reported that adiponectin levels were significantly correlated with ALT, γ GT and ALP, independently of sex, age, BMI and insulin resistance, thus suggesting a wider role for adiponectin in the maintenance of liver integrity (23). However, the correlation between adiponectin and liver transaminases was not confirmed in a study carried out in patients with advanced cirrhosis, who showed increased adiponectin levels, while a direct correlation was found between plasma adiponectin and reduced liver hemodynamics, thus suggesting that increased adiponectin in these patients may be due to the alteration of liver hemodynamics rather than to liver function (24).

Interestingly, an inverse correlation between ALT and adiponectin was reported in an obese population which is known to have a high prevalence of NAFLD (8). However, a direct demonstration in humans of reduced adiponectin levels in NAFLD and NASH based on histologic findings has never been reported previously. It is interesting to note that control and NAFLD group were strictly matched for BMI and age and both groups were similarly overweight. Nevertheless, NAFLD patients had a markedly reduced plasma adiponectin and were more insulin resistant than controls. Moreover, on regression analysis, adiponectin was inversely correlated with HOMA, thus supporting the role of adiponectin in the link between insulin resistance and NAFLD. However, *in vivo* regulation of plasma adiponectin is not fully understood, and it is still under debate whether circulating adiponectin is upregulated by meals (25, 26). Since changes of adiponectin due to meals may be a confounding factor, we chose to measure adiponectin levels after the overnight fast. However, it is not possible to exclude that NAFLD patients have also an altered response of adiponectin to meals and/or an altered 24-h pattern of circulating peripheral or portal adiponectin secretion.

In conclusion, our study reported a lower plasma adiponectin in NAFLD patients that is inversely correlated with insulin resistance. These data support a role for adiponectin in protection against liver injury, in keeping with the hypothesis that an imbalance between proinflammatory and anti-inflammatory cytokines may have a pathogenic role in the development of liver damage in NAFLD. Finally, a novel cross-talk between adipose tissue and liver is emerging that may

act as a major player in the link between metabolic syndrome and fatty liver disease.

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