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RESEARCH ARTICLE

Pattern of Expression of Adiponectin Receptors in Human Liver and its Relation to Nonalcoholic Steatohepatitis

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Abstract
Background Adiponectin has antisteatosis–anti-inflammatory properties and its circulating levels are reduced in nonalcoholic steatohepatitis (NASH).
Methods To assess the role of adiponectin in NASH, we measured expression of adiponectin gene (*APM1*) and receptors (*AdipoR1*/*AdipoR2*) in liver and subcutaneous and visceral fat in subjects with biopsy-proven NASH or pure steatosis (PS). In 103 subjects undergoing gastric bypass or elective abdominal surgery (17 with normal liver histology (C), 52 with PS, and 34 with NASH), RNA was extracted from tissue samples, and quantification of *APM1*, *AdipoR1*, and *AdipoR2* was carried out by real-time polymerase chain reaction.
Results In NASH vs C, circulating adiponectin levels (3.6 [2.4] vs 5.3[4.3] μg/ml, median[interquartile range], $p<0.05$) and adiponectin concentrations, *APM1*, *AdipoR1*, and *AdipoR2* expression in visceral fat were all reduced ($p\leq0.03$). These differences disappeared when adjusting for obesity. In contrast, liver *AdipoR1* (1.40 [0.46] vs 1.00 [0.32] of controls) and *AdipoR2* expression (1.20 [0.41] vs 0.78 [0.43]) were increased in NASH, and group differences were statistically significant ($p<0.0001$ for *AdipoR1* and $p=0.0001$ for *AdipoR2*). Results for PS were generally intermediate between NASH and C. Liver receptor expression was reciprocally related to circulating adiponectin ($rho=-0.42$, $p<0.003$ for *AdipoR1* and $rho=-0.26$, $p<0.009$ for *AdipoR2*). In multivariate models adjusting for sex, age, fasting plasma glucose, and obesity, liver enzymes levels were directly related to both *AdipoR1* and *AdipoR2* expression in liver.
Conclusion In obese patients with NASH, adiponectin receptors are underexpressed in visceral fat—as a likely correlate of obesity—but overexpressed in liver, possibly as a compensatory response to hypoadiponectinemia, and positively associated with liver damage.

Keywords Adiponectin · Adiponectin receptors · Obesity · Nonalcoholic steatohepatitis

Abbreviations

APM1	adiponectin gene
ApN	adiponectin protein
AdipoR1 and *AdipoR2*	adiponectin receptor gene
GADPH	glyceraldehyde-3-phosphate dehydrogenase
HPRT	hypoxantine-phosphorybosyl-transferase
Ob	obesity

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NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
PS	pure steatosis
PCR	polymerase chain reaction
SAT	subcutaneous adipose tissue
VAT	visceral adipose tissue

Introduction

Nonalcoholic fatty liver disease (NAFLD) is an important cause of chronic liver failure in individuals without significant alcohol consumption [1]. NAFLD includes a spectrum of syndromes ranging from pure steatosis (PS) to nonalcoholic steatohepatitis (NASH) to cirrhosis and hepatocellular carcinoma [1–4]. While NASH is a condition with a high potential to progress to cirrhosis, PS appears to follow a more benign course, with little or no progression to further liver damage [2, 5–9].

Obesity, diabetes mellitus, hypertension, and dyslipidemia are common clinical risk factors associated with PS and NASH [1]. Although the pathogenesis of NAFLD remains largely unknown, insulin resistance has been implicated as a key mechanism in its pathogenesis and is also believed to contribute to its progression [10–14]. However, while there is an evidence that most patients with NAFLD have some degree of insulin resistance, not every individual with insulin resistance develops NASH. This suggests that insulin resistance may be necessary but is clearly not sufficient for the development of NAFLD. Numerous substances, mainly released by adipocytes, are thought to contribute to insulin resistance and subclinical inflammation. Among these adipocytokines, adiponectin stands out for its insulin-sensitizing anti-inflammatory role [15–17]. Thus, adiponectin knockout mice exhibit an enhanced pattern of hepatic fibrosis induced by carbon tetrachloride, while in wild-type mice adiponectin administration prevents liver fibrosis following carbon tetrachloride exposure [17]. In patients with type 2 diabetes [16], plasma adiponectin concentrations are inversely related to hepatic fat content. Moreover, Hui et al. [18] have shown a direct relationship between hypoadiponectinemia and NASH independent of insulin resistance [18].

Two adiponectin receptors (AdipoR1 and AdipoR2) have been identified and found to be expressed in various tissues [19]. AdipoR1 has a high affinity for globular adiponectin, whereas AdipoR2 has intermediate affinity for the full-length and the globular adiponectin isoform. Adiponectin receptors are expressed in the liver; the level of their expression in NAFLD is controversial, however. Thus, Kaser et al. [20] have reported reduced expression of both adiponectin receptors in 13 NASH patients (as compared to nine control subjects that included PS), while Vuppalanchi et al. [21] found higher mRNA levels of *AdipoR2* in NASH. On the other hand, the expression of adiponectin and both its receptors is known to be reduced in adipose tissue—especially visceral adipose tissue—in obesity [22–24], which is a risk factor for NAFLD. Also, circulating levels of total adiponectin (including all three major isoforms) correlate with the expression of the adiponectin gene in visceral fat despite the fact that subcutaneous fat is the major source of circulating adiponectin.

In the present study, we measured the expression of the adiponectin gene (*APM1*) and both its receptors (*AdipoR1* and *AdipoR2*) in the liver and subcutaneous and visceral fat tissue in a relatively large group of subjects with biopsy-proven PS or NASH together with measurements of adiponectin concentrations in plasma and adipose tissue.

Methods and Procedures

Subjects and study protocol One hundred and three subjects, with or without type 2 diabetes, on the waiting list for bariatric surgery (subjects with severe obesity) or minor elective abdominal surgery (cholecystectomy or antireflux procedures) were enrolled to participate in the study. Diabetic subjects regularly taking more than one oral hypoglycemic agent or on insulin were excluded. Subjects with an estimated alcohol consumption>20 g/day, end-stage renal disease, cardiac failure, hepatitis B virus or hepatitis C virus, autoimmune chronic hepatitis, Epstein-Barr virus infection, non-organ-specific autoantibodies (antimitochondrial, antinuclear, antismooth muscle, antiliver microsomal), thyroid disease, hereditary defects (as judged from serum iron, transferrin saturation, ferritin, ceruloplasmin, α-1-antitripsin levels), and use of potentially hepatotoxic drugs (amiodarone, corticosteroids, tamoxifen, methotrexate, oral contraceptives, antidepressants) were excluded.

All patients underwent the metabolic study 2 weeks before surgery (having stopped any antidiabetic drug at least 1 week previously) and were instructed not to exercise for 48 h before the study as well as before surgery. For the metabolic study, all subjects were examined in the morning after an overnight (12–14 h). Peripheral blood samples were obtained for determination of lipid profile, glucose, insulin, and adiponectin concentrations. Then, a 75-g oral glucose tolerance test was performed, with sampling every 15 min within the first hour and every 30 min for the following 2 h, for measurement of plasma glucose and insulin. Impaired glucose tolerance and diabetes were classified according to the ADA criteria [25]. Biopsies of liver tissue, abdominal subcutaneous (SAT) and visceral (VAT) adipose tissue, were obtained during surgery. All subjects gave informed,

written consent, and the protocol was approved by the local Ethical Committee.

Analytical determinations Plasma glucose concentration was measured on a Beckman Glucose Analyzer 2 (Beckman, Fullerton, CA, USA). Fasting concentrations of serum total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were determined as described elsewhere (Synchron CX4, Beckman Instruments, Inc., Brea, CA, USA). Liver enzymes (aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyl transpeptidase, alkaline phosphatase) were measured by an enzymatic method (Synchron CX 4, Beckman Instruments, Inc., Fullerton, CA, USA). Plasma insulin was measured by an insulin-specific radioimmunoassay and adiponectin by enzyme-linked immunosorbent assay (ELISA; Linco Research, St. Charles, MO, USA).

Liver histology Liver biopsy specimens were fixed in formalin, routinely processed, sectioned, and stained with hematoxylin–eosin, silver for reticulin, Perls for iron, periodic acid Schiff after diastase digestion for activated macrophages, orcein for copper-associated protein and elastic fibers, Masson's trichome for connective tissue, and ubiquitin immunostain when Mallory bodies are difficult to identify. All biopsies were evaluated by a single hepatologist—blinded to the clinical or biochemical findings—for the following parameters: degree of steatosis, ballooning degeneration of hepatocytes, hepatocytes containing Mallory material, lobular and portal inflammation, extent and location of collagen fibrosis, and iron deposition. The degree of steatosis was assessed and graded—in terms of estimated percentage of tissue occupied by fat vacuoles—as follows: 0=none; 1=up to 5%; 2=6–33%; 3=34–66%; 4=>66%. Diagnosis of simple steatosis or NASH was performed using Brunt classification [26]. Simple steatosis (PS) was defined as steatosis without significant lobular or portal inflammation, ballooning degeneration, or fibrosis. Steatohepatitis (NASH) was defined as steatosis involving up to 66% of lobules accompanied by ballooning degeneration, lobular or portal inflammation, Mallory bodies and zone III of Rappaport pericellular, and sinusoidal or perivenular fibrosis. No cases of cirrhosis were found in the population studied.

Total RNA isolation and cDNA preparation Specimens of SAT and VAT tissue (1–2 g) and liver (20 mg) were collected in RNA-Later and stored at −20°C. The adipose tissue was cut into pieces of 600 mg; after removing RNA-Later, total RNA was isolated using a mixed protocol: Trizol and RNeasy Midi Kit (Qiagen, Hilden, Germany) in order to obtain a larger amount of purified RNA. Total RNA was extracted from liver tissue using RNeasy Mini Kit (Qiagen, Hilden, Germany), after tissue-lyzer homogenization (Tissue Lyser, Qiagen, Hilden,, Germany). RNA was quantified by measuring absorbance at 260 and 280 nm. RNA integrity was then checked by visual inspection of the two ribosomal RNAs, 18S and 28S, on 1% agarose gel. Average yields of total RNA were 20± 1 μg/g of adipose tissue and 5±1 μg/mg of liver. Isolated RNA was stored at −80°C until quantification of target mRNAs. For first-strand cDNA synthesis, 1 μg total RNA was reverse-transcribed in a 20-μl volume using random hexamers as primers, according to the manufacturer's instructions (first-strand cDNA Synthesis Kit for reverse-transcription polymerase chain reaction (PCR), AMV, Roche, Indianapolis, IN, USA).

Quantification of APM1, AdipoR1, and AdipoR2 gene expression Relative quantification of mRNAs was performed by real-time PCR using a Light-Cycler instrument (Roche Diagnostics, Mannheim, Germany) as previously described [24]. In brief, primers for the target genes (*AdipoR1*, NM 015999: forward 5′-TTCTTCCTCATGGCTGTGATGT-3', reverse 5′-AAGAAGCGCTCAGG-AATTCG-3', 71 bp; *AdipoR2*, NM 024551: forward 5'-CCACCACCTTGCTT CATCTA-3', reverse 5'-GATACTGAGGGGTGGCAAAC-3', 97 bp; *APM1*, AB012165: forward 5′-TGGTGA GAAGGGTGAGAA-3' and reverse 5'-AGATCTTGG TAAAGCGAATG-3', 221 bp) and the reference gene (hypoxantine-phosphoribosyl-transferase, *HPRT*, M 26434: forward 5′-TGCTGACCTGCTGGATTAC-AT-3', reverse 5′-TTGCGACCTTG-ACCATCTTT-3', 260 bp) were designed in our laboratory. One microliter of cDNA was brought to a final volume of 20 μl in a glass capillary containing 1X Light-Cycler-FastStar DNA Master SYBR Green I mix (Roche Diagnostics), 4.5 mM MgCl2, and 0.3 μM of primers (Proligo, Paris, France) for *APM1*, *AdipoR1*, and *AdipoR2* and 0.5 μM for HPRT. PCR was performed with 10 min of initial denaturation and then 40 cycles with 10 s at 95°C (denaturation), 7 s at 58–60°C (annealing), and 13 s at 72°C (extension). The last cycle at 40°C for 15 s. Fluorescent data were acquired at the end of each extention phase. After amplification, a melting curve analysis from 65°C to 95°C with heating rate of 0.1°C/s with a continuous fluorescence acquisition was constructed.

Real-time PCR for *AdipoR1* and *AdipoR2* expression in the liver was also repeated using as reference gene glyceraldehyde-3-phosphate dehydrogenase (*GADPH*: forward 5'-TGCACCACCAACTGCTTAGC-3', reverse 5'GGCATGGACTGTGGTCATGAG-3'). First of all, we pooled total RNA from ten control subjects; cDNA was synthesized and diluted in a threefold serial dilution (1:3 to 1:27). Then, at each dilution target (*APM1*, *AdipoR1*, and *AdipoR2*), reference genes (*HPRT*) were amplified in triplicate to construct standard curves for each gene.

Standard curves were accepted only if the slope was approximately −3, with an r value>0.98, and were used to estimate PCR efficiency. Following that, a sample of the pool used for the standard curve (diluted 1:3) was run together with the cDNA sample of each subject as a "calibrator" in order to correct for single experiment efficiency and to check cDNA stability. The cDNA from the 103 subjects was quantified in triplicate; the mean value for each sample was used. For the relative quantification of samples, the Light-Cycler Relative Quantification Software was utilized. Normally, *HPRT* was used as the reference gene in SAT, VAT, and liver, and *GADPH* was used as further control. As there were no detectable differences in index gene expression evaluated with *HPRT* or *GAPHD*, data were normalized to *HPRT*. Results were expressed as the target-to-reference ratio of each sample, normalized by the target-to-reference ratio of the calibrator. In the present data, the coefficient of variation in measurements for the target gene in each sample ranged 0–10%. No amplification was detected for *APM1* in the liver.

Adipocyte isolation and measurement of intracellular adiponectin concentrations In a subgroup of subjects (12 controls, 15 PS, and eight NASH), mature adipocytes were isolated as described by Grohmann et al. [27]. The adipose tissue was washed three times in Hank's balanced salt solution, cut into 1-mm^3 pieces, and digested with 3 ml of 4 mg/ml type II collagenase in Hank's balanced salt solution for 60 min at 37°C. Fragments of tissue still remaining after this treatment were removed with forceps. The mature adipocytes were separated from the stromal fraction by centrifugation at 90×g for 3 min and were carefully removed to a sterile Eppendorf containing 500 μl of lysis buffer (10 mM Tris–Cl, 50 mM NaCl, 5 mM EDTA, 15 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovanadate, and 1% Triton X-100 with freshly added 1% phenylmethylsulfonyl fluoride at the time of use). Cells were then lysed via repeated passaging through a 21-gauge needle and the fat cake at the top of the sample was carefully removed; samples were then frozen at −20°C. Samples were processed in batch, and adiponectin (ApN) concentrations were determined using a standard ELISA assay (Linco Research, St. Charles, MO, USA); adipose tissue ApN concentrations were corrected for milligram of total protein.

Statistical analysis Results are expressed as mean±SD or median [interquartile range], for variables with normal or nonnormal distribution, respectively. Categorical variables were compared by the χ^2 test; continuous variables were analyzed by one-way analysis of variance (ANOVA); the Bonferroni–Dunn test was used for post hoc analysis. Variables with nonnormal distribution were log-transformed for statistical testing. Univariate associations were tested with Spearman coefficient, *rho*. Multivariate analysis was performed by using general linear models including both continuous and categorical variables; results are given as the standardized regression coefficient (*st.r*).

Results

Subjects with NASH had higher BMI, fasting plasma glucose and insulin concentrations, HOMA index, and liver enzymes—and lower HDL cholesterol—than subjects with normal liver histology (controls). BMI was also higher in subject with PS compared to controls (Table 1). There were no diabetic subjects in the control group, whereas the prevalence of diabetes was 22% and 39% in subjects with PS and NASH, respectively (p=0.01). With regard to the use of antidiabetic agents, no significant difference was found between PS (15% was on antidiabetic treatment: 75% with metformin and 25% with sulfonylureas) and NASH (24% was on treatment: 67% with metformin and 33% with sulfonylureas); likewise, the PS and NASH groups did not differ in relation to antihypertensive treatment (ACE inhibitor, calcium antagonist, β-blocker, and diuretic drugs). Plasma adiponectin levels tended to be reduced in PS and were markedly depressed in NASH. Furthermore, plasma adiponectin was not significantly different between men and women (3.6 [2.6], 4.3 [3.8] μg/ml, respectively) or in postmenopausal vs premenopausal women (age 20–40 years 4.9 [2.2] μg/ml, age 41–50 years 5.3 [3.3] μg/ml, age>50 years 7.7 [5.9] μg/ml). Adiponectin was negatively related to BMI (*rho*=−0.34, p=0.001), fasting plasma insulin (*rho*=−0.48, p=0.0001), AST (*rho*=−0.32, p<0.01), and ALT (*rho*=−0.34, p<0.005) and directly related to HDL cholesterol (*rho*=0.35, p<0.01).

In the liver, *AdipoR1* expression was 1.03 [0.28] (range 0.52–1.65), 1.18 [0.40] (range 0.73–2.29), and 1.40 [0.46] (range 0.81–2.58) in controls, PS, and NASH, respectively (p<0.0001). Similarly, *AdipoR2* was 0.78 [0.41] (range 0.41–1.32), 0.94 [0.34] (range 0.38–2.6), and 1.20 [0.41] (range 0.55–2.61), p=0.0001 in controls, PS, and NASH (Fig. 1). Eighty-five percent of subjects with NASH had liver *AdipoR1* and *AdipoR2* expression higher than the mean of the control group. Furthermore, 20% of subjects with NASH had expression levels of adiponectin receptors higher than the maximum level of controls.

Receptor expression did not differ by gender (*AdipoR1* 1.18 [46] vs 13 [38]; *AdipoR2* 13 [44] vs 95[41], men vs women, or between premenopausal and postmenopausal women (details not shown). The between-group differences were still statistically significant (p<0.01 for *AdipoR1* and

Table 1 Clinical and metabolic parameters according to liver histology

	Controls (n=17)	PS (n=52)	NASH (n=34)	p
Gender (M/F)	5/12	7/45	13/21	<0.04
Age (years)	41±11	43±11	40±10	ns
BMI (kg/m^2)	27.2 [17.2]	44.9 [12.6]*	48.8 [6.2]*	0.0001
SBP(mmHg)	123±19	129±15	132±16	ns
DBP(mmHg)	77±14	78±12	82±10	ns
Total cholest. (mg/dl)	186±30	204±34	197±44	ns
HDL cholest. (mg/dl)	52±7.8	47±11.5	44±11.6	ns
Triglycerides (mg/dl)	109±29	162±95	152±94	ns
FPG (mg/dl)	89±11	105±30	127±50*	0.001
2-h PG (mg/dl)	125±43	135±46	159±57	ns
FPI (pmol/l/l)	51 [70]	101 [71]	119 [140]	ns
2-h PI (pmol/l)	481 [370]	450 [497]	592 [645]	ns
HOMA	2.2 [2.5]	4.1 [3.3]	6.0 [7.2]**	0.02
AST (U/l)	15.5 [5.0]	20.5 [7.0]	35.0 [16.0]*	<0.0001
ALT (U/l)	15.0 [8.0]	22.0 [10.0]	45.0 [34.5]*	<0.0001
γGT (U/l)	16.5 [20.0]	21.0 [17.0]	39.0 [36.2]*	ns
Plasma ApN (μg/ml)	5.3 [4.3]	4.5 [4.3]	3.6 [2.4]*	<0.03
WBC (×10^3 per cubic millimeter)	6.93±1.1	6.99±1.2	6.9±1.2	ns

SBP systolic blood pressure, *DBP* diastolic blood pressure, *cholest.* cholesterol, *FPG* fasting plasma glucose, *2-h PG* 2-h postload plasma glucose, *FPI* fasting plasma insulin, *2-h PI* 2-h postload plasma insulin, *ApN* adiponectin, *WBC* white blood cell
*p≤0.03 vs controls; **p≤0.02 vs PS by Bonferroni–Dunn test

p=0.04 for *AdipoR2*) after adjusting for gender, age, BMI, and fasting plasma glucose levels. In the whole dataset, *AdipoR1* was significantly more expressed than *AdipoR2* (by ~25%, p<0.0001) and was strongly related to it (*rho*= 0.67, p<0.0001). Plasma adiponectin was reciprocally related to *AdipoR1* (*rho*=−0.42, p<0.003) and *AdipoR2* (*rho*=−0.26, p<0.009). Furthermore, AST levels were positively related to liver expression of *AdipoR1* (*rho*= 0.34, p<0.001) and *AdipoR2* (*rho*=0.27, p<0.01), and ALT concentrations were significantly related to *AdipoR1* (*rho*= 0.33, p=0.001) and *AdipoR2* (*rho*=0.29, p<0.01). In multivariate models adjusting for sex, age, BMI, and fasting plasma glucose liver enzymes levels were directly related to both *AdipoR1* and *AdipoR2* expression (Fig. 2).

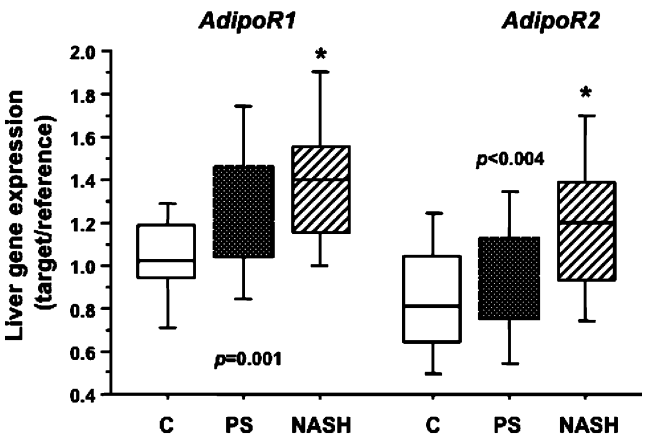

Fig. 1 *AdipoR1* and *AdipoR2* expression in subjects with normal liver histology (*C*), liver steatosis (*PS*), or steatohepatitis (*NASH*). Boxes plot median, 95% confidence intervals, and range. p values by ANOVA, *asterisks* indicate p<0.001 vs controls (*C*)

In SAT, *APM1*, *AdipoR1* and *AdipoR2* expression, and tissue adiponectin content did not significantly differ by liver histology (Table 2). In contrast, in VAT *APM1*, *AdipoR1*, and *AdipoR2* expression were all significantly lower in NASH—and tended to be lower in PS—as compared to controls (Table 2). Moreover, in a subgroup of subjects (12 controls, 15 PS, and eight NASH), VAT tissue adiponectin content was significantly diminished in association with both PS and NASH. All the above differences disappeared when VAT data were adjusted for BMI.

Discussion

The main findings of the present study are: (1) adiponectin receptors were expressed in the liver at statistically higher levels in subjects with NASH—and, to a lesser, statistically nonsignificant extent, PS—as compared to subjects with normal liver histology; this is in striking contrast with the situation in the visceral adipose tissue of the same subjects, where adiponectin receptor mRNA as well as expression of the adiponectin gene and tissue adiponectin levels were

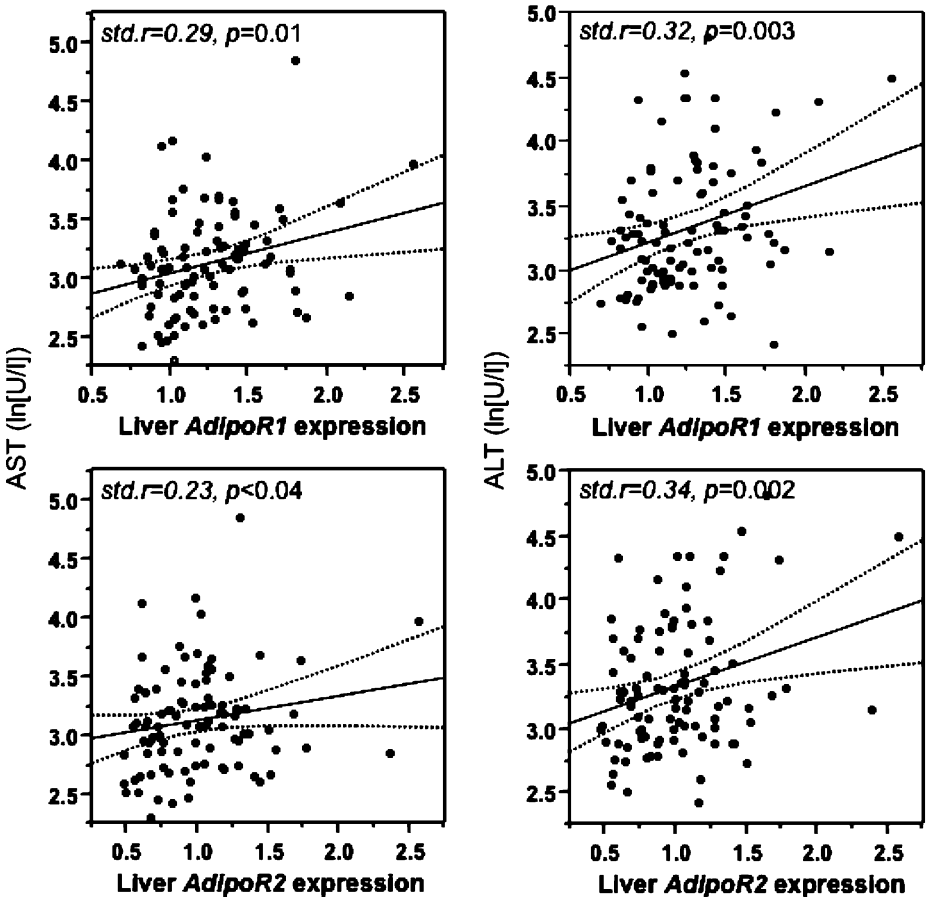

Fig. 2 Association of *AdipoR1* and *AdipoR2* expression with liver enzymes (AST and ALT). The individual data plotted are the residuals from a multiple regression model adjusting for gender, age, fasting plasma glucose, and BMI. *Full line* = best fit; *dotted lines* = 95% confidence intervals; *std.r* = standardized regression coefficient

markedly reduced in NASH; (2) increased hepatic adiponectin receptor expression was coupled with lower circulating adiponectin levels and raised serum liver enzymes. These findings require specification.

There is an increasing interest concerning the role of adiponectin and its receptors in liver disease, but data from animal and human studies are few and sometimes conflicting. In a recent paper by Neumeier et al. [28], the expression of adiponectin receptors was evaluated in six rats fed standard chow vs six rats fed a high-fat diet (a model of fatty liver disease): in the latter group, systemic adiponectin was found to be reduced; *AdipoR1* mRNA and protein levels were similar between groups, whereas *AdipoR2* mRNA was increased in high-fed diet rats. On the contrary, in the same study, mice treated with bile duct ligation (a model of cirrhotic liver) showed increased

Table 2 Adiponectin concentrations, adiponectin gene and receptor gene expression in subcutaneous and visceral adipose tissue

	Controls	PS	NASH	*p*
Subcutaneous fat				
APM1	1.13 [1.71]	0.79 [1.18]	0.97 [0.98]	ns
AdipoR1	1.26 [0.45]	1.04 [0.84]	1.10 [0.58]	ns
AdipoR	4.77 [10.4]	4.69 [9.52]	4.72 [6.71]	ns
Adiponectin (μg/g)	20 [21]	18 [181]	21 [25]	ns
Visceral fat				
APM1	0.67 [0.66]	0.58 [0.75]	0.47 [0.38]*	0.02
AdipoR1	0.75 [0.62]	0.58 [0.38]*	0.49 [0.31]*	0.03
AdipoR2	0.76 [1.42]	0.58 [0.44]	0.49 [0.26]*	0.02
Adiponectin (μg/g)	51 [98]	20 [20]*	25 [23]*	0.003

p values by ANOVA
*p=0.02 vs controls by Bonferroni–Dunn test

systemic adiponectin with suppressed receptor expression as compared to control mice.

The pattern of liver expression of adiponectin and its receptors in our study group is at complete variance with the results reported by Kaser et al. [20]. In our data, *APM1* expression in the liver was undetectable; *AdipoR1* mRNA was significantly higher than *AdipoR2* mRNA, and both *AdipoR1* and *AdipoR2* expression were progressively increased from controls to NASH through PS. On the other hand, Vuppalanchi et al. [21] could not detect any *APM1* signal in the liver and reported higher *AdipoR2* expression in NASH than controls, in accord with our study. Methodological differences—such as the use of a different reference gene in the real-time PCR analysis—are unlikely to explain the discrepancy between our results and those of Kaser et al. [20] because we obtained the same pattern of results when using *GADPH* instead of *HPRT*. Indirect support for our findings in the liver comes from the observation that expression of adiponectin and its receptors was reduced in the visceral fat tissue of the same subjects, which fully confirms previous studies in human adipose tissue [22–24]. Perhaps more importantly, the differences in adipose tissue expression between patients and controls disappeared when adjusting for BMI; in contrast, the group differences in liver expression persisted after controlling for gender, age, BMI, and fasting plasma glucose. In addition, hepatic receptor expression was positively associated with serum liver enzymes independently of gender, age, BMI, and glycemia. Thus, not only did the expression levels differ between NASH vs normal in opposite directions in liver and fat—enhanced in the former, reduced in the latter—but the changes in NASH liver were independent of obesity. This suggests that adiponectin receptors expression in the liver may be related, directly or indirectly, to liver damage.

Secondly, circulating adiponectin was, if weakly, inversely related to the hepatic expression of both its receptors, thus strengthening the results of Vullapanchi R et al. [21]. In general, it is difficult to extrapolate adiponectin gene expression or tissue levels to circulating levels because: (a) at the whole body level, secretion depends on the product of tissue-specific secretion rate by total fat mass, (b) different mixtures of globular and full-length protein could be released by different tissues, and (c) gene expression levels may not quantitatively translate into secretion rates if there are posttranslational effects or autocrine–paracrine interactions. Nevertheless, it is of note that, in the whole dataset, both plasma adiponectin levels and adiponectin gene expression in subcutaneous fat—which is the most abundant depot—were reciprocally related to BMI, indicating that an expanded fat mass expresses less *APM1* and releases less protein into the bloodstream [22, 29]. In the visceral fat depot of our NASH subjects, there was a consensual marked reduction in *APM1* expression and tissue protein concentrations and an inverse correlation between circulating adiponectin levels and liver enzymes in agreement with other studies [21, 30, 31]. Therefore, it could be speculated that liver receptors were upregulated as a result of particularly low hormone levels flowing to the organ through the portal vein from a fat depot profoundly impoverished of adiponectin. This negative feedback loop between hormone levels and receptor density—similar to that of insulin [32]—would represent a compensatory response aimed at preserving adiponectin actions on the liver. There may, however, be additional factors at play. The fact that liver receptor expression was increased in NASH—and proportional to serum enzyme levels—even after controlling for BMI suggests either that in a fatty inflamed liver adiponectin receptors are overexpressed inherently, i.e., because of a shift in the cellular phenotype, or that receptor overexpression is somehow causally related to liver damage and dysfunction. The current data do not allow distinguishing between these two alternatives, and further investigation is needed to clarify the significance of adiponectin receptor overexpression in hepatic tissues.

All in all, our findings are not incompatible with the notion that adiponectin plays a protective role in the development and progression of NAFLD [15, 18]. Adiponectin has antilipogenic properties that may protect non-adipose tissues, such as the liver, against lipid accumulation [15]. In vitro studies have shown that adiponectin exerts anti-inflammatory effects, such as inhibition of nuclear factor κB activation [33] and suppression of macrophage function [34, 35]. In animal models of alcoholic steatohepatitis, exogenous adiponectin quenches hepatic inflammation and decreases the hepatic expression and plasma concentrations of tumor necrosis factor α [15]. There are data to show that adiponectin has an anti-inflammatory hepatoprotective effects in humans with fatty liver attributable to metabolic factors [15, 18]. Some studies have recently reported low circulating adiponectin levels associated with NASH independent of insulin resistance [15, 16, 36]. The paradigm may be, however, more complex.

In conclusion, we report that: (a) obese patients with NASH have significantly lower levels of serum adiponectin and adiponectin content and expression as well as adiponectin receptor expression in visceral adipose tissue as a likely consequence of obesity; (b) in NASH patients, adiponectin receptor expression in the liver is increased regardless of obesity and is directly related to liver enzymes. This evidence confirms that the adiponectin system could play an important role in the development of liver damage in subjects with metabolic disease.

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References

1. McCollough AJ. Update on non-alcoholic fatty liver disease. J Clin Gastroenterol. 2002;34:255–62.
2. Matteoni CA, Younossi ZM, Gramlich T, et al. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. Gastroenterol 1999;116:1413–9.
3. Falck-Ytter Y, Younossi ZM, Marchesini G, et al. Clinical features and natural history of non-alcoholic steatosis syndromes. Semin Liver Dis. 2001;21:17–26.
4. Bugianesi E, Leone N, Vanni E, et al. Expanding the natural history of non-alcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. Gastroenterol 2002;123:134–40.
5. Teli MR, James OF, Burt AD, et al. Natural history of non-alcoholic fatty liver: a follow-up study. Hepatology 1995;22:1714–9.
6. Bacon BR, Farahvash MJ, Janney CG, et al. Nonalcoholic steatohepatitis: an expanded clinical entity. Gastroentolo 1994;107:1103–9.
7. Day CP, James OFW. Steatohepatitis: a tale of two "hits"? Gastroenterol 1998;114:842–5.
8. James OF, Day CP. Non-alcoholic steatohepatitis (NASH): a disease of emerging identity and importance. J Hepatol. 1998;29:495–501.
9. Angulo P, Keach JC, Batts KP, et al. Independent predictors of liver fibrosis in patients with non-alcoholic steatohepatitis. Hepatology 1990;30:1356–62.
10. Chitturi S, Farrell GC. Etiopathogenesis of non-alcoholic steatohepatitis. Semin Liver Dis. 2001;21:27–41.
11. Younossi Z, Gramlich T, Matteoni C, et al. Non-alcoholic fatty liver disease in patients with type II diabetes. Clin Gastro Hepatol. 2004;2:262–5.
12. Sanyal AJ, Campbell-Sargent C, Mirshahi F, et al. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. Gastroenterol 2001;120:1183–92.
13. Chitturi S, Abeygunasekera S, Farrell GC, et al. NASH and insulin resistance: insulin hypersecretion and specific association with the insulin resistance syndrome. Hepatology 2002;35:373–9.
14. Marchesini G, Brizi M, Morselli-Labate AM, et al. Association of non-alcoholic fatty liver disease with insulin resistance. Am J Med. 1999;107:450–5.
15. Xu A, Wang Y, Keshaw H, et al. The fat-derived hormone adiponectin alleviates alcoholic and non-alcoholic fatty liver diseases in mice. J Clin Invest. 2003;112:91–100.
16. Bajaj M, Suraamornkul S, Piper P, et al. Decreased plasma adiponectin concentrations are closely related to hepatic fat content and hepatic insulin resistance in pioglitazone-treated type 2 diabetic patients. J Clin Endocrinol Metab. 2004;89:200–6.
17. Kamada Y, Tamura S, Kiso A, et al. Enhanced carbon tetrachloride-induced liver fibrosis in mice lacking adiponectin. Gastroenterol 2003;125:1796–807.
18. Hui JM, Hodge A, Farrell GC, et al. Beyond insulin resistance in NASH: TNF-alpha or adiponectin? Hepatology 2004;40:46–54.
19. Yamauchi T, Kamon J, Ito Y, et al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 2003;423:762–9.
20. Kaser S, Moschen A, Cayon A, et al. Adiponectin and its receptors in non-alcoholic steatohepatitis. Gut 2005;54:117–21.
21. Vuppalanchi R, Marri S, Kolwankar D, et al. Is adiponectin involved in the pathogenesis of non-alcoholic steatohepatitis? J Clin Gastroenterol. 2005;39:237–42.
22. Lihn AS, Bruun JM, He G, et al. Lower expression of adiponectin mRNA in visceral adipose tissue in lean and obese subjects. Mol Cell Endocrinol. 2004;219:9–15.
23. Statnick MA, Beavers LS, Conner LJ, et al. Decreased expression of apM1 in omental and subcutaneous adipose tissue of humans with type 2 diabetes. Int J Exp Diabetes Res. 2000;1:81–8.
24. Nannipieri M, Bonotti A, Anselmino M, et al. Pattern of expression of adiponectin receptors in human adipose tissue depots and its relation to the metabolic state. Int J of Obes. July 2007;31:1843–8.
25. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care. 2002;25:S5–20.
26. Brunt EM, Janney CG, Bisceglie AM, et al. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol. 1999;94:2467–74.
27. Grohmann M, Sabin M, Holly J, et al. Characterization of differentiated subcutaneous and visceral adipose tissue from children: the influence of TNF-a and IGF-1. J Lipid Res. 2005;46:93–103.
28. Neumeier M, Hellerbrand C, Gabele E, et al. Adiponectin and its receptors in rodent models of fatty liver disease and liver cirrhosis. World J Gastroenterol. 2006;12:5490–4.
29. Hoffstedt J, Arvidsson E, Sjolin E, et al. Adipose tissue adiponectin production and adiponectin concentration in human obesity and insulin resistance. JCEM 2004;89:1391–6.
30. Lu JY, Su TC, Liu YH, et al. Lower plasma adiponectin is correlated with alanine aminotransferase independent of metabolic factors and hepatitis B virus carrier status. Intern Med J. 2007;37:365–71.
31. Bugianesi E, Pagotto U, Manini R, et al. Plasma adiponectin in nonalcoholic fatty liver is related to hepatic insulin resistance and hepatic fat content, not to liver disease severity. J Clin Endocrinol Metab. 2005;90:3498–504.
32. Okabayashi Y, Maddux BA, McDonald AR, et al. Mechanisms of insulin-induced insulin-receptor downregulation. Decrease of receptor biosynthesis and mRNA levels. Diabetes 1989;38:182–737.
33. Ouchi N, Kihara S, Arita Y, et al. Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappa B signaling through a cAMP-dependent pathway. Circulation 2000;102:1296–301.
34. Yokota T, Oritani K, Takahashi I, et al. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. Blood 2000;96:1723–32.
35. Ding X, Saxena NK, Lin S, et al. The role of leptin and adiponectin. A novel paradigm in adipocytokine regulation of liver fibrosis and stellate cell biology. Am J Pathol. 2005;166:1655–69.
36. Sargin H, Sargin M, Gozu H, et al. Is adiponectin level a predictor of nonalcoholic fatty liver disease in nondiabetic male patients? World J Gastroenterol. 2005;11:5874–7.