Title

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Journal

Obesity (Silver Spring, Md.), 18(12)

ISSN

1930-7381

Authors

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Publication Date

2010-12-01

DOI

10.1038/oby.2010.95

Peer reviewed

Increased Soluble Leptin Receptor Levels in Morbidly Obese Patients With Insulin Resistance and Nonalcoholic Fatty Liver Disease

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The adipocyte hormone, leptin has been demonstrated to have profibrogenic actions *in vitro* and in animal models. However, no correlation was found between plasma leptin levels and fibrosis stage in humans. Thus, our aim was to study whether soluble leptin receptor (SLR) or free leptin index (FLI; calculated as the ratio of leptin to SLR), may correlate better with the features of metabolic syndrome and with the histological grade and stage of nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH). We studied a population (n = 104) of morbidly obese patients undergoing bariatric surgery. Data including BMI, type 2 diabetes mellitus, hypertension, and hyperlipidemia were obtained. Plasma fasting leptin and SLR, fasting glucose and insulin were measured, and homeostasis model of assessment insulin resistance ($HOMA_{IR}$) index and FLI were calculated. All patients had intraoperative liver biopsies. Leptin levels correlated with the BMI. The multiple regression analysis indicated that increasing HOMA and decreasing FLI were predictors of steatosis in the liver ($P < 0.0003$). SLR levels were positively correlated with the presence of diabetes mellitus and the stage of fibrosis. In conclusion, increased SLR levels in morbidly obese patients with diabetes are correlated with the stage of liver fibrosis, and may reflect progressive liver disease.

Obesity (2010) **18,** 2268–2273. doi:10.1038/oby.2010.95

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases, and even though the exact prevalence is not known, it is estimated to affect about 30% of the general population in urban communities (1) and about 70% of obese individuals (2). Per definition, it occurs in individuals who consume minimal amounts of alcohol, and it is frequently associated with the features of the metabolic syndrome including obesity, insulin resistance/diabetes, hypertension, and hyperlipidemia (3,4). The spectrum of liver histology in NAFLD ranges from steatosis to nonalcoholic steatohepatitis (NASH) which is characterized by macrovesicular steatosis with various degrees of lobular inflammation, hepatocyte ballooning, and predominantly centrilobular fibrosis. About 15% of patients with NAFLD will develop NASH, which ultimately may cause cirrhosis and hepatocellular carcinoma (5). Leptin is an adipokine, and is implicated in the pathogenesis of NASH, due to its important modulatory effects on the regulation of body weight, appetite and in preventing lipid accumulation in the skeletal muscle, pancreas, and liver (6,7). Obesity is strongly associated with increased circulating leptin concentrations. However, obese patients often develop leptin resistance which aggravates the metabolic defects (8). The exact mechanism of the development of leptin resistance in obese patients is unknown but it is suspected that sequestration of circulating free leptin may play a role (8). Of interest, leptin has been shown to have profibrogenic effects on the liver in *in vitro* and in animal models (9–11), suggesting a potential role of this hormone in the pathogenesis and progression of fibrosis in NASH. Despite this, no study in human subjects has found a convincing correlation between circulating leptin levels and stage of fibrosis and the role of leptin in hepatic fibrogenesis in

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Received 4 August 2009; accepted 31 March 2010; published online 6 May 2010. doi:10.1038/oby.2010.95

humans are still debated (12–14). In this context, increasing attention has been devoted to the soluble leptin receptor (SLR), a major leptin-binding protein in the serum, generated by the proteolytic cleavage of the ectodomain of the membrane-bound leptin receptor. However, the exact role of SLR in the regulation of leptin bioavailability remains unknown (15,16). SLR is thought to be produced mainly by the liver (17) therefore changes of SLR in the circulation may reflect the pathogenic changes during NASH development.

In this study, we described a positive correlation of SLR with the histological stage of NAFLD/NASH in diabetic, morbidly obese patients. These data may have future implications with regard to the role of SLR in the development leptin resistance in NASH.

METHODS AND PROCEDURES

Patients

Data and samples were collected from December 2005 to March 2008 on patients undergoing bariatric surgery at University of California Davis Medical Center (Sacramento, CA), who consented for the study. A questionnaire was answered by the potential participants at the initial evaluation regarding their liver disease history. All patients had intraoperative surgical core liver biopsies of the lateral right hepatic lobe, according to a standard surgical protocol. We reviewed individual medical charts for demographics, medical history and physical examination, preoperative laboratory data, and risk factors for the metabolic syndrome (BMI, hypertension, hyperlipidemia, type 2 diabetes mellitus). Patients with clinical or radiological evidence of cirrhosis were not considered candidates for bariatric surgery and, therefore were not included in our analysis. Potential etiologies for other chronic liver diseases were excluded by medical history, hepatitis A, B, and C serologies, histology, ceruloplasmin, transferrin saturation, and α-1 antitrypsin level. Diagnosis of hypertension was made according to the guideline set by Joint National Committee 7 (18). Diagnosis of hyperlipidemia was made following the guideline set by National Cholesterol Education Program III (19). The BMI was calculated as the weight in pounds divided by the square of the height in feet. The study was conducted in accordance with the ethical standards of the Helsinki Declaration of 1975 (as revised in 1983). This study was approved by University of California Davis Medical Center institutional review board (protocol #200513532-3).

Biochemical assays

Once informed consent was obtained, all participants had blood samples drawn before the surgery (09:00–11:00 AM), after an overnight fast. The blood samples were then immediately put on ice, and plasma was obtained by centrifugation at 1,400 *g* for 10 min at 4 °C. The plasma was spun again at 2,700 *g* for 10 min at 4 °C, and the recovered plasma was stored in aliquots at −80 °C. The EDTA-plasma was analyzed for glucose, insulin, total leptin (RIA; Linco Research, St Charles, MO), and SLR (ELISA; Diagnostic Systems Laboratories, Webster, TX). The homeostasis model of assessment insulin resistance ($HOMA_{IR}$) was used as an index of insulin resistance. $HOMA_{IR}$ was calculated using the standard formula: (fasting glucose (ml/dl) × fasting insulin (μU/ml))/405. Free leptin index (FLI) index was calculated as the ratio of leptin to SLR.

Histology

The liver biopsy specimens were reviewed blindly by a hepatopathologist for the presence of macrovesicular steatosis, fibrosis, and neutrophilic lobular inflammation according to the Brunt's scoring system (20). Steatosis was graded on a four-point scale: grade 0: steatosis involving <10% of hepatocytes; grade 1: steatosis up to 30%; grade 2: steatosis between 30 and 60%; and grade 3: steatosis >60%. Lobular inflammation was graded on a four-point scale based on inflammatory foci per 20×: grade 0; 1 with 1–2 foci; 2 with 3–4 foci; 3 with >4 foci. Fibrosis was staged on a five-point scale, stage 0: no fibrosis; stage 1: zone 3 perisinusoidal fibrosis; stage 2: perisinusoidal fibrosis and periportal fibrosis; stage 3: focal or extensive bridging fibrosis; stage 4: cirrhosis.

Data analysis

Data are presented as mean values ± s.d. for continuous variables and frequencies for categorical variables. Bivariate relationship for measures with grade of steatosis and stage of fibrosis was evaluated using χ^2-test for categorical variables. Spearman correlation coefficient and its *P* value for significance of correlation were calculated to assess the magnitude and direction of an association between two given measures based on their ordered ranks. For data that were highly skewed, we applied a natural log transformation to achieve normality and significance testing on a log-transformed scale. The relative risks of developing steatosis and fibrosis associated with clinical measures were estimated using the odds ratios and their corresponding 95% confidence intervals through step-wise multiple logistic regression analysis. We used 5% as the cutoff for the probability of entering a variable in the model and removing a variable from the model. The probabilities were calculated using the Wald test. Deviance residuals were used to evaluate the goodness-of-fit, and likelihood ratio test was performed to compare the reduced model with the full model. All reported *P* values are based on two-sided tests. A two-sided *P* value <0.05 was considered significant. All analyses were performed using SAS statistical software (SAS Institute, Cary, NC).

RESULTS

Clinical features and the metabolic syndrome

One hundred seventy nine patients listed for bariatric surgery have consented for this study. One hundred and four of the enrolled patients had satisfactory liver specimens, and their plasma samples were collected appropriately to be included in the analysis. Forty-nine patients were excluded because of suboptimal liver specimens (subcapsular specimens, burn artefacts), 24 because the plasma samples were not adequate for SLR analysis, and 2 patients did not undergo the bariatric procedure after intraoperative evidence of cirrhosis. Baseline demographic and metabolic features are summarized in **Table 1**. Females were 76% of the studied group. There was no significant age difference between the two genders. The mean BMI of the whole group was 46.2 ± 5.8, and females had significantly lower BMI than males (45.5 ± 6 vs. 48.3 ± 4.8, $P = 0.03$). Hyperlipidemia and hypertension were the most represented features of metabolic syndrome, both present in >60% of patients, and they were significantly correlated ($r = 0.2$, $P = 0.01$). The mean $HOMA_{IR}$ index was 7.1 ± 10.6.

Association between the plasma leptin, SLR, and FLI values and the clinical parameters

The mean plasma leptin concentration was 43.7 ± 20.1 ng/ml. Leptin levels tended to be higher in women than men (46.3 ± 18.2 vs. 35.6 ± 23.6 ng/ml, $P = 0.02$) and were significantly correlated with the BMI ($r = 0.37$, $P < 0.0001$), similarly to the data from previous reports (13,21,22). SLR was positively correlated with the presence of diabetes ($r = 0.24$, $P = 0.01$), whereas its derivative, the FLI was negatively correlated with this parameter ($r = -0.2$, $P = 0.002$) (**Table 2**). However, we did not find any significant correlations of SLR with the fasting glucose or insulin concentrations.

Table 1 Clinical and metabolic features of the 104 enrolled patients

Age	43.2 ± 9.2
Gender (F/M)	79/25 (76/24%)
BMI	46.2 ± 5.8
Hypertension	63 (61%)
Diabetes type 2	39 (37.5%)
HOMA index	7.1 ± 10.6
Hyperlipidemia	65 (63%)
Total cholesterol (mg/dl)	184 ± 40
LDL cholesterol (mg/dl)	115 ± 32.8
HDL cholesterol (mg/dl)	40 ± 11.8
Triglyceride (mg/dl)	157 ± 84
Leptin (ng/ml)	43.7 ± 20.1
Soluble leptin receptor (U/ml)	38.4 ± 28.4
Free leptin index (ng/U)	1.5 ± 1.1
Grade of steatosis	
<10%	23 (22%)
10–29%	39 (38%)
30–60%	38 (37%)
>60%	4 (4%)
Grade of inflammation	
0	45 (43.3%)
1	49 (47.1%)
1.5	5 (4.8%)
2	5 (4.8%)
Stage of fibrosis	
0	81 (78%)
1	5 (5%)
3	18 (17%)

Values are presented as mean ± s.d. or frequency (%).
HOMA, homeostasis model of assessment; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Association between the SLR/FLI values and the histological grade/stage of liver disease

The main histological findings are summarized in Table 1. The majority of the patients had a steatosis degree between 10 and 60%. Eighty-three percentage of patients had stage 0–1 fibrosis, the remaining 17% presented with stage 3 fibrosis. We did not observe any differences in the distribution of the disease severity between men and women. Steatosis was positively correlated with the $HOMA_{IR}$ index ($r = 0.39$, $P < 0.0001$) and with the presence of diabetes ($r = 0.24$, $P = 0.015$). In contrast to previous studies reporting a positive association between steatosis and circulating leptin concentrations (12,21); we did not observe any significant correlation between these two parameters in this patient population.

As for the fibrosis stage, we found a positive correlation to the SLR values ($r = 0.22$, $P = 0.02$) (Figure 1), whereas its derivative, the FLI exhibited a negative trend toward a correlation with fibrosis, as expected ($r = -0.18$, $P = 0.06$). For the FLI, there was a negative trend toward a correlation with steatosis as well, however, the latter did not achieve statistical significance, either ($P = 0.054$). In agreement with previous reports (12,13), there was no correlation between fibrosis and plasma leptin levels. A positive correlation was seen between the $HOMA_{IR}$ index and the stage of fibrosis ($r = 0.24$, $P = 0.01$), corroborating previous studies showing that diabetes and insulin resistance are the factors most closely associated with the severity of liver disease, in particular in patients with normal alanine aminotransferase (23,24).

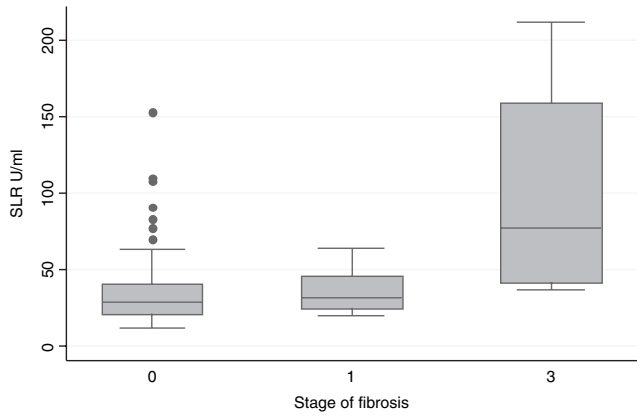

Figure 1 Plasma soluble leptin receptor (SLR) concentrations and the stage of fibrosis. The levels of SLR were positively correlated with the stage of fibrosis in morbidly obese patients undergoing bariatric surgery ($r = 0.22$, $P = 0.02$). The dots represent patients with extreme values in the high end.

The degree of inflammation was not significantly correlated with any of the clinical or biochemical variables we examined, but it was positively correlated with the stage of fibrosis ($r = 0.3$, $P = 0.001$), consistent with a role for inflammation in the pathogenesis of hepatic fibrosis (25). Significant correlations are summarized in Table 2.

Stepwise regression analysis was performed to ascertain the predictors of steatosis among several independent variables. The independent variables included age, gender, BMI, presence of hypertension, hyperlipidemia, fasting plasma glucose, fasting plasma insulin, $HOMA_{IR}$ index, leptin, SLR, and FLI. All the independent variables were initially considered in the multiple regression analysis. The multiple logistic regression analysis revealed that the elevated insulin and decreasing levels of FLI were the significant predictors of steatosis after adjusting for the other variables. Backward elimination was carried out to determine which variables should be excluded from the fitted model using the likelihood ratio test. The final model included two significant predictors, elevated insulin ($P = 0.0005$), and decreasing levels of FLI ($P = 0.031$). The analysis also suggested that the combination of fasting insulin and decreasing levels of FLI still remained significant predictors of steatosis (overall $P = 0.0021$), after adjusting for age and gender (Table 3).

Similarly, stepwise logistic regression analysis was performed to identify independent predictors for the stage of fibrosis after controlling for other variables. The multiple logistic regression

Table 2 Bivariate correlations between features of the metabolic syndrome, leptin, soluble leptin receptor (SLR), and free leptin index (FLI) and the histological parameters of nonalcoholic fatty liver disease severity as calculated by the Spearman correlation (coefficient and *P* value)

Parameters	Fibrosis	Steatosis	Inflammation	BMI	DM2	HOMA	Hypertension	Hyperlipidemia
Steatosis	0.13 0.18							
Inflammation	0.3 **0.001**	0.12 0.22						
BMI	0.13 0.17	–0.03 0.76	0.05 0.54					
DM2	0.17 0.07	0.24 **0.015**	0.02 0.84	0.1 0.3				
HOMA	0.24 **0.01**	0.39 **<0.0001**	0.008 0.92	0.09 0.33	0.43 <0.0001			
Hypertension	0.01 0.89	0.10 0.32	–0.002 0.97	–0.01 0.85	0.38 **<0.0001**	0.26 **0.007**		
Hyperlipidemia	–0.003 0.97	0.13 0.20	–0.03 0.7	0.01 0.9	0.16 0.09	0.13 0.17	0.24 **0.01**	
Leptin	–0.06 0.52	–0.17 0.09	–0.004 0.96	0.37 **<0.0001**	–0.15 0.1	0.002 0.98	–0.1 0.09	–0.02 0.78
SLR	0.22 **0.02**	0.12 0.23	0.03 0.73	–0.1 0.28	0.24 **0.01**	–0.08 0.4	0.09 0.32	–0.02 0.79
FLI	–0.18 0.06	–0.16 0.10	–0.007 0.93	0.3 **0.002**	–0.29 **0.002**	0.04 0.64	–0.14 0.13	0.01 0.89

Significant *P* values are depicted in bold.
DM2, type 2 diabetes mellitus; HOMA, homeostasis model of assessment.

Table 3 Multiple logistic regression analysis for predictors of steatosis

Factors	Odds ratio	95% CI	*P* value	Overall *P* value
Final model				
FLI	0.58	0.35–0.95	0.0316	0.0003
Insulin	3.98	1.83–8.66	0.0005	
Final model adjusted for age and gender				
FLI	0.61	0.36–1.02	0.0613	0.0021
Insulin	3.78	1.72–8.31	0.0009	

Steatosis was considered as a categorical variable, and four categories of steatosis were included in the analysis (<10% steatosis, 10–29%, 30–60%, >60%).
CI, confidence interval; FLI, free leptin index.

Table 4 Multiple logistic regression analysis for predictors of fibrosis

Factors	Odds ratio	95% CI	*P* value	Overall *P* value
Final model				
FLI	0.31	0.14–0.71	0.0053	0.0002
BMI	1.10	1.00–1.21	0.0439	
Inflammation	4.48	1.76–11.40	0.0017	
Final model adjusted for age and gender				
FLI	0.34	0.14–0.82	0.0169	0.0002
BMI	1.09	0.98–1.21	0.1055	
Inflammation	4.93	1.79–13.55	0.0020	

Fibrosis was considered as a categorical variable, and the stages of fibrosis included in the analysis were stages 0, 1, and 3. One case of stage 2.5 was incorporated with stage 3.
CI, confidence interval; FLI, free leptin index.

analysis identified that BMI ($P = 0.0439$), inflammation ($P = 0.0017$), and decreasing level of FLI ($P = 0.0053$) were the significant independent predictors for the stage of fibrosis. When we further adjusted for age and gender, the combination of decreasing FLI and inflammation remained significantly associated with fibrosis in this patient population (overall $P = 0.0002$) (Table 4).

DISCUSSION

In this study, we found that SLR was positively correlated with the stage of fibrosis in morbidly obese patients with insulin resistance or diabetes, whereas its derivative, FLI was a negative predictor. There was also a positive correlation between inflammation and the progression of fibrosis, supporting previous observations (26,27). SLR, thought to be mainly derived from the liver (17), is one of the main leptin-binding proteins in the plasma and it may regulate the levels of biologically active free leptin (28). SLR is generated by the proteolytic cleavage of the membrane-bound leptin receptor, ObRe (ectodomain shedding), a process which is regulated under various physiological and pathophysiological conditions (29,30). Matrix metalloproteinases, especially the ADAMTs (A Disintegrin and Metalloproteinase with Thrombospondin-1-like domains) group, were reported to be involved in the cleavage of SLR (31,32). The proteolytic activity of ADAMTs has been correlated to stellate cells activation and, *in vivo*, to liver fibrogenesis

(32,33). As activated stellate cells in the liver produce matrix metalloproteinases, it is conceivable that the rise in plasma SLR may reflect matrix metalloproteinase activity and fibrogenesis in the liver. Indeed, recent studies in patients with cirrhosis from various etiologies described increasing SLR and lower FLI, similarly to our study (34). As leptin is thought to be a profibrogenic cytokine *in vitro*, one may expect increasing free leptin levels in cirrhotic patients. There could be several reasons for this contradiction: first, we do not know whether the peripheral SLR/leptin levels reflect the sinusoidal concentrations, second, leptin may play different roles at the initiation and progression phases of fibrosis. Detailed mechanistic studies are required to elucidate these aspects.

Increasing plasma SLR levels found in obese, diabetic patients could point to the development of leptin resistance (15). Leptin resistance is an important phenomenon as it causes further dysregulation of metabolic and inflammatory pathways, worsening insulin resistance, ultimately leads to progressive organ injury in obese patients with the metabolic syndrome (35). Recently it was described that in the liver-specific insulin receptor knockout mice there was about an 80-fold increase in circulating SLR, pointing to the importance of intact insulin signaling in the liver in the modulation of leptin receptor expression (36).

Our study was consistent with previous findings concerning the positive correlation between leptin and BMI (21). According to earlier data, we have not seen any significant correlation between the plasma leptin levels and fibrosis (12,21,37). Our study population consisted of morbidly obese adult patients with insulin resistance, who are frequently asymptomatic but have under recognized metabolic complications and liver disease. The patients in our study were relatively younger, with generally less advanced liver damage than usually is seen in patients referred to the hepatology clinic, yet our patients represent a significant study group. Patients referred with NASH often have well-established cirrhotic stage disease, and studying markers contributing to disease activity in this group sometimes is not feasible. Also, in our study there were more women than men, whereas other studies have reported a higher prevalence of NASH in men (1,38). We recognize that larger studies with more patients with advanced NASH are needed to validate these findings.

Future studies at a molecular level are needed to define the causal correlation between markers of fibrogenic activity, inflammation, and the circulating SLR in patients with obesity and NASH.

DISCLOSURE

The authors declared no conflict of interest.

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