

Lys656Asn polymorphism of leptin receptor, leptin levels and insulin resistance in patients with non alcoholic fatty liver disease

R. ALLER¹, D.A. DE LUIS², O. IZAOLA², M. GONZÁLEZ SAGRADO²,
R. CONDE², D. PACHECO², M.C. VELASCO², H.F. OVALLE²

¹Department of Gastroenterology, Hospital Clínico Universitario de Valladolid (Spain)

²Center of Investigation of Endocrinology and Clinical Nutrition, Medicine School and Unit of Investigation, Hospital Rio Hortega, Valladolid University, Valladolid (Spain)

Abstract. – **Background:** Some studies have pointed to a role of leptin and insulin resistance in pathogenesis of non alcoholic fatty liver disease (NAFLD). The aim of our study was to investigate the influence of Lys656Asn polymorphism LEPR gene on the histological changes, insulin resistance and leptin levels in overweight patients.

Material and Methods: A population of 76 patients with NAFLD was recruited in a cross sectional study. A biochemical analysis of serum was measured. Genotype of LEPR gene Lys656Asn was studied.

Results: Nineteen patients (25%) had the genotype Lys656Asn and 4 patients genotype Asn656Asn (mutant type group) and 53 patients (69.7%) Lys656Lys (wild type group). Body mass index, weight, fat mass, waist circumference, waist to hip ratio, glucose levels and HOMA-IR were higher in mutant than wild type group. LEPR polymorphism is in any way related with liver lesions. The multivariate analysis adjusted by age, sex, BMI and genotype showed an independently association of lobular inflammation 4.19 (CI95%: 1.37-12.77), portal inflammation 1.97 (CI95%: 1.05-3.74) and steatosis 9.23 (CI95%: 1.47-57.83) with HOMA. Liver steatosis was associated with leptin levels (1.09 (CI95%: 1.06-1.18)), too.

Conclusion: Lys656Asn polymorphism of LEPR gene is associated with obesity parameters, insulin resistance and glucose levels in patients with NAFLD. In logistic regression analysis, only insulin resistance was associated with portal inflammation, lobular inflammation and steatosis; liver steatosis was related with leptin levels, too.

Key Words:

Lys656Asn polymorphism of leptin receptor, Biopsy, Insulin resistance, Leptin.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a liver disease characterized by elevated serum aminotransferases levels and accumulation of fat in liver accompanied by inflammation and necrosis resembling alcoholic hepatitis in the absence of heavy alcohol consumption^{1,2}. Obesity is considered the most important risk factor and insulin resistance has been associated with fat liver and NAFLD, too³. The association with insulin resistance and obesity has also suggested that NAFLD should be considered part of the metabolic syndrome⁴. NAFLD is a complex metabolic condition in which both lifestyle and genetic factors have a pathogenic role.

Adipose tissue secretes several bioactive proteins, or adipocytokines, that regulate hepatic and peripheral glucose metabolism. Leptin, an adipocyte-derived hormone, has been showed to play an important role in the pathogenesis of NAFLD. For example, Hui et al⁵ suggested that raised serum leptin levels in non alcoholic steatohepatitis (NASH) may be a reflection of the failure of leptin to stimulate hepatic lipid turnover, it could be called as a leptin resistance status.

The discovery of mutations in the leptin gene and its receptor in rodent models of obesity and in rare cases of morbid obesity patients indicates that leptin functions as an afferent signal in a negative feedback that regulates energy balancing⁶. This has been demonstrated in some severely obese humans who are homozygous for a mutation in the leptin receptor, who have very high leptin levels⁷. These mutations are extremely rare and cannot be responsible for obesity in the general population. Different polymorphisms in leptin receptor gene

have been studied with unclear results⁸. The polymorphism on codon 656 produces a change in charge, making this change a possibility to be functional in this receptor. Leptin receptors have been identified in hypothalamic regions and in peripheral tissues such as pancreatic beta cells⁹, muscle, adipose tissue and hepatocytes. Leptin has been shown to be able to modulate insulin secretion and action through these receptors. Moreover, Lu et al¹⁰ have demonstrated that polymorphism of LEPR gene 3057 probably contributes to the onset of NAFLD by regulating insulin sensitivity. In other study¹¹, it has been demonstrated that LEPR polymorphism Lys109Arg is not related with NAFLD. However, as far as we know, the relationship of LEPR polymorphism Lys565Asn with NAFLD has not been evaluated.

The aim of our study was to investigate the influence of Lys656Asn polymorphism LEPR gene on the histological changes, insulin resistance and leptin levels in overweight patients.

Subjects and Methods

Subjects

During the period January 2007-July 2010, consecutive 76 Caucasian obese subjects were recruited for this study. The exclusion criterias were alcohol consumption, diabetes mellitus, fasting glucose intolerance, hepatitis B, C, cytomegalovirus, Epstein Barr infections, nonorgan-specific autoantibodies, medication (blood-pressure lowering medication and statins) and hereditary defects (iron and copper storage diseases and alpha 1-antitrypsin deficiency). The study was approved by the institutional Ethics Committee. Patients signed an informed consent.

Liver Biopsies

The diagnosis of NAFLD was confirmed by percutaneous liver biopsy performed in all subjects with a 1.6 mm Menghini-type biopsy needle. Liver samples were routinely processed, sectioned, and stained with hematoxylin-eosin and Manson's trichome. All biopsies were studied by the same liver pathologist (T.A.G.). Histology was analysed using the Brunt classification¹². Steatosis was graded as follows: mild (<33% of hepatocytes affected); moderate-severe (≥33% of hepatocytes affected). The Brunt system also includes as grading: portal inflammation, ballooning, lobular inflammation and staging fibrosis:

Stage 1: zone 3 perivenular perisinusoidal/pericellular fibrosis, focal or extensive;

Stage 2: as above with focal or extensive periportal fibrosis;

Stage 3: bridging fibrosis, focal or extensive;

Stage 4: cirrhosis. In our study, fibrosis variable was divided as absent or presence and inflammation (portal and lobular) stage was divided as mild or moderate-severe.

Procedures

In all subjects, blood sampling was performed in the fasting state at 08.00 hours. After clotting, blood samples were centrifuged for 10 min at 8000 rpm. Serum was stored at -80°C for later determination. Basal glucose, insulin, insulin resistance (HOMA), total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides and leptin blood levels were analyzed.

Serum total cholesterol and triglyceride concentrations were measured by enzymatic colorimetric assay (Technicon Instruments, Ltd., New York, N.Y., USA), while HDL cholesterol was determined enzymatically in the supernatant after precipitation of other lipoproteins with dextran sulfate-magnesium. LDL cholesterol was calculated using Friedewald formula.

Plasma glucose levels were measured by using an automated glucose oxidase method (Glucose analyser 2, Beckman Instruments, Fullerton, CA, USA). Insulin was measured by RIA (RIA Diagnostic Corporation, Los Angeles, CA, USA) with a sensitivity of 0.5 mUI/L (normal range 0.5-30 mUI/L)¹³ and the homeostasis model assessment for insulin resistance [(HOMA-IR)= (fasting blood glucose x fasting insulin)/22.5)] was calculated using these values¹⁴.

Leptin was measured by ELISA (Diagnostic Systems Laboratories, Inc., Minneapolis, MN, USA) with a sensitivity of 0.05 ng/ml and a normal range of 10-100 ng/ml, interassay coefficients of variation were less than 15%¹².

Anthropometric Measurements

Height and weight were measured in light clothing, body weight was measured to an accuracy of 0.1 kg and body mass index was calculated as body weight/(height²). Waist and hip circumferences were measured and waist-to hip ratio (WHR) was calculated by the ratio of waist to hip circumference. Tetrapolar body electrical bioimpedance was used to determine body composition. An electric current of 0.8

mA and 50 kHz was produced by a calibrated signal generator (Biodynamics Model 310e, Seattle, WA, USA).

Genotyping of LEPR Gene Polymorphism

Genomic DNA was extracted from peripheral blood leucocytes by the phenol chloroform method. Oligonucleotide primers and probes were designed with the Beacon Designer 4.0 (Premier Biosoft International®, Los Angeles, CA, USA). The polymerase chain reaction (PCR) was carried out with 250 ng of genomic DNA, 0.5 ul of each oligonucleotide primer (primer forward: 5'-GCA GTT CCT ATG AGA GGA CC-3'; primer reverse: 5'-AAA TTG GGA ATA CCT TCC AAA GT-3'), and 0.25 µL of each probes (wild probe: 5'-Fam-AGT GAC ATT TTT CTC CTT TTT CAT AGT ATC-Tamra-3') and (mutant probe: 5'-Hex-AGT GAC ATT TTT CTC GTT TTT CAT AGT AT- Tamra -3') in a 25 µL final volume [Termociclador iCycler IQ (Bio-Rad®), Hercules, CA, USA]. DNA was denaturized at 95°C for 3 min; this was followed by 50 cycles of denaturation at 95°C for 15 s, and annealing at 59.3° for 45 s). The PCR were run in a 25 µL final volume containing 12.5 µL of IQTM Supermix (Bio-Rad®, Hercules, CA, USA) with hot start Taq DNA polymerase. Hardy-Weinberg equilibrium was assessed.

Statistical Analysis

Sample size was calculated to detect differences over 1 units of HOMA with 90% power and 5% significance (n=70). The statistical analysis was performed for the combined Lys656Asn and Asn656Asn as a mutant group and wild type G308G as second group (Dominant model). The results were expressed as mean ± standard deviation. The distribution of variables was analyzed with Kolmogorov-Smirnov

test. Continuous variables with normal distribution were analyzed with a two-tailed, paired Student's-*t* test. Non-parametric variables were analyzed with the Mann-Whitney U test. Categorical variables were analyzed with the chi-square test, with Yates correction as necessary, and Fisher's test. Multivariate stepwise logistic regression was used to select the variables independently associated with histological variables of liver biopsies, all the models adjusted by age, sex, body mass index and genotype. Hardy-Weinberg equilibrium was assessed. A *p*-value under 0.05 was considered statistically significant. All statistical analyses were conducted by using the SPSS statistical package ver. 15.0 (SPSS Software, Chicago, IL, USA).

Results

Univariate Analysis

Seventy six patients gave informed consent and were enrolled in the study. The mean age was 43.3±12.1 years and the mean BMI 33.7±7.9 with 50 males (65.8%) and 26 females (34.2%). Nineteen patients (6 females/13 males) (25%) had the genotype Lys656Asn and 4 patients (1 female/3 male) genotype Asn656Asn (mutant type group) and 53 patients (12 females/39 males) (69.7%) Lys656Lys (wild type group).

The anthropometric characteristics are summarized in Table I. BMI, weight, fat mass, waist circumference and waist to hip ratio were higher in mutant than wild type group.

Table II shows the differences in cardiovascular risk factors. Glucose levels and HOMA were higher in mutant type group than wild type group. No differences were detected in other biochemical parameters.

Table I. Changes in anthropometric variables.

Parameters	Lys656Lys (N = 53)	Lys656Asn and Asn656Asn (N = 23)	<i>p</i>
Age (years)	43.1 ± 11.1	43.8 ± 14.6	0.85
BMI	32.2 ± 8.8	36.9 ± 11.5	0.02
Weight (kg)	89.6 ± 20.3	102.3 ± 36.7	0.01
Fat mass (kg)	22.9 ± 7.4	28.6 ± 11.5	0.001
Waist circumference (cm)	98.1 ± 13	105.4 ± 14.9	0.002
Waist to hip ratio	0.93 ± 0.1	0.98 ± 0.1	0.02

p < 0.05 (statistical differences). BMI: Body mass index.

Table II. Classical cardiovascular risk factors.

Parameters	Lys656Lys (N = 53)	Lys656Asn and Asn656Asn (N = 23)	<i>p</i>
Glucose (mg/dl)	103.1 ± 22	118.0 ± 31.1	0.02
Total ch. (mg/dl)	191.5 ± 51.6	207.4 ± 48.8	0.67
LDL-ch. (mg/dl)	118.8 ± 43.8	131.4 ± 43.4	0.48
HDL-ch. (mg/dl)	54.9 ± 25.4	49.3 ± 11.4	0.54
TG (mg/dl)	136.1 ± 87	133.5 ± 87.8	0.91
Insulin (mUI/L)	14.0 ± 10.3	15.2 ± 6.9	0.61
HOMA-IR	3.6 ± 2.5	4.5 ± 2.4	0.02
Leptin (ng/mL)	35.6 ± 29.1	46.8 ± 35.1	0.11

Ch.: Cholesterol. TG: Triglycerides. HOMA-IR: Homeostasis model assessment. *p* < 0.05 (statistical differences).

Table III shows the histological lesions in relation to both genotypes. Patients with mutant type group presented similar ratios of mild/moderate-severe portal inflammation, mild/moderate-severe portal inflammation, mild/moderate-severe portal steatosis and absent/presence of fibrosis than patients with wild type group. LEPR genotype did not influence on histological changes of liver in these NAFLD patients.

In the analysis by histological lesions, patients with moderate-severe steatosis had higher levels of HOMA (2.7±1.4 vs 5.0±1.4: *p* < 0.05), leptin (29.3±20.1 vs 48.5±30.1 pg/ml: *p* < 0.05), insulin (10.2±4.1 vs 18.2±11.1 mUI/L: *p* < 0.05), fat mass (22.6±8.7 vs 25.4±8.2 kg: *p* < 0.05), weight (84.3±19.2 vs 100.4±30.2 kg: *p* < 0.05) and BMI (29.9±6.5 kg/m² vs 36.5±11.2 kg/m²: *p* < 0.05) than patients with mild steatosis. Patients with moderate-severe lobular inflammation had higher

levels of HOMA (3.3±2.5 vs 4.4±2.5: *p* < 0.05), leptin (26.6±21.1 vs 45.9±36.1 pg/ml: *p* < 0.05), insulin (12.9±7.7 vs 15.5±7.1 mUI/L: *p* < 0.05), fat mass (19.7±8.3 vs 26.3±7.7 kg: *p* < 0.05), weight (85.8±17.2 vs 97.2±31.2 kg: *p* < 0.05) and BMI (30.3±6.5 vs 35.6±11.1 kg/m²: *p* < 0.05) than patients with mild lobular inflammation. Patients with moderate-severe portal inflammation had higher levels of HOMA (3.5±2.4 vs 5.2±2.6: *p* < 0.05), insulin (13.2±9.1 vs 18.1±8.1 mUI/L: *p* < 0.05), leptin (30.3±25.1 vs 64.5±46.1 pg/ml: *p* < 0.05), fat mass (23.3±8.5 vs 26.6±8.4 kg: *p* < 0.05), weight (89.1±19.2 kg vs 106.1±41.2: *p* < 0.05) and BMI (32.1±8.2 vs 38.4±13.1 kg/m²: *p* < 0.05) than patients with mild portal inflammation. Patients with liver fibrosis had higher levels of HOMA (3.6±2.3 vs 5.5±3.1: *p* < 0.05), insulin (13.5±9.3 vs 19.5±9.1 mUI/L: *p* < 0.05), leptin (31.9±26.1 vs 71.7±46.1

Table III. Histological parameters.

Parameters	Lys656Lys (N = 53)	Lys656Asn and Asn656Asn (N = 23)
Steatosis		
Mild	33 (68.8%)	15 (31.3%)
Moderate-Severe	20 (71.4%)	8 (28.6%)
Lobular inflammation		
Mild	22 (66.7%)	11 (33.3%)
Moderate-severe	31 (63.1%)	12 (27.9%)
Portal inflammation		
Mild	41 (70.7%)	18 (29.3%)
Moderate-severe	12 (70.6%)	5 (29.4%)
Fibrosis		
Absent	44 (69.8%)	19 (30.2%)
Presence	9 (68.3%)	4 (30.7%)

Chi square test, no statistical differences. (%) frequencies in each genotype group, the amount of mild and moderate-severe damage is 100%.

pg/ml: $p < 0.05$), fat mass (23.3 ± 8.5 vs 26.6 ± 8.4 kg: $p < 0.05$), weight (88.5 ± 19.8 vs 115.9 ± 43.2 kg: $p < 0.05$) and BMI (31.5 ± 7.2 vs 43.2 ± 15.1 kg/m²: $p < 0.05$) than patients without fibrosis.

Multivariate Analysis

Choosing each histological parameter as a dependent variable, multivariate stepwise logistic regression was used to select other variables independently associated. The multivariate analysis adjusted by age, sex, BMI and genotype with the dependent variable (portal inflammation) showed that HOMA remained in the model, with an increase of the probability to develop moderate-severe inflammation of 1.97 (CI95%: 1.05-3.741) with each increase of one unit on HOMA levels. The model with the dependent variable (lobular inflammation) showed that HOMA remained in the model, with an increase of the probability to develop moderate-severe inflammation of 4.19 (CI95%: 1.37-12.77) with each increase of one unit on HOMA levels. The next model with the dependent variable (steatosis) showed that HOMA and leptin levels remained in the model, with an increase of the probability to develop moderate-severe steatosis of 9.23 (CI95%: 1.47-57.83) with each increase of one unit on HOMA levels and 1.09 (CI95%: 1.06-1.18) with each increase of each ng/ml of leptin levels. The multivariate analysis with the dependent variable (fibrosis) did not showed independent variables.

Discussion

The present study demonstrates that Lys656Asn polymorphism of LEPR gene is associated with obesity parameters, insulin resistance and glucose levels in patients with NAFLD. In logistic regression analysis, only insulin resistance was associated with portal inflammation, lobular inflammation and steatosis; liver steatosis was related with leptin levels, too.

Previous studies have been detected association between polymorphism on codon 656 and cardiovascular risk factors, as our study. In postmenopausal women with intolerance of glucose (IGT), associations were found with Lys565Asn for fasting insulin, as well as in response to an oral glucose tolerance test (OGTT)¹⁵. In other study with nondiabetic obese patients¹⁶, Lys565Asn genotype was associated with high levels of insulin and HOMA. Moreover, in dia-

betic obese patients, Lys565Asn genotype was associated with high levels of HOMA, C reactive protein, leptin and fat mass¹⁷. Leptin has been shown to be able to modulate insulin secretion and action through these receptors. *In vitro*, a direct leptin-induced inhibition of basal and glucose-stimulated insulin secretion was shown in pancreatic islets under conditions of high concentrations or prolonged exposure⁹. Leptin was also shown to be able to modulate insulin-induced activities in adipocyte, hepatocyte and muscle cells^{18,19}. These effects could be mediated by both peripheral mechanisms related to leptin receptors in peripheral organs and central nervous system pathways, probably through the autonomic nervous system²⁰. However, although our results show a clear relationship between insulin resistance and hepatic lesions of NAFLD, as well as a link between the LEPR polymorphism and insulin resistance, there was no relationship between polymorphism and hepatic lesions. This lack of association does not have a clear explanation, may be due to confounding factors not tested, like other genetic or environmental variables.

Moreover, Marchesini et al²¹ demonstrated a closely correlation between insulin resistance (HOMA) and NAFLD, too. Other Authors have been detected this relation using the clamp technique²²⁻²⁴ with results supporting our conclusions. The nature of the connection between insulin resistance and hepatic steatosis remains unclear²⁵. In obese patients, the primary abnormality may be genetically induced insulin resistance, with a secondary increase of serum triglyceride levels due to enhance of peripheral lipolysis. The resulting hepatic supply of fatty acids and insulin may increase triglyceride deposition in the liver^{24,25}.

The role of leptin on hepatic lesions of NAFLD is less clear. In animal experiments, obese and diabetic ob/ob mice, which are leptin deficient, developed steatohepatitis but not liver fibrosis when fed a methionine–choline-deficient (MCD) diet²⁶, but an obese/diabetic experimental model (db/db mice) showed development of marked liver fibrosis for NASH and suggested an important role for *LEPR* in the pathogenesis of NASH²⁷, while leptin injections attenuate their fatty livers and metabolic abnormalities²⁸. Patients with type 2 diabetes mellitus complicated with NAFLD had higher plasma leptin levels than those without NAFLD¹⁰, suggesting that leptin may play an important role in the pathogenesis on NAFLD. Lep-

tin therapy may reduce liver enzymes, body mass index (BMI), hepatic fat content and histological features of steatohepatitis²⁹.

Common variants in the human LEPR gene have been demonstrated to associate with insulin resistance, obesity and leptin levels¹⁵⁻¹⁷, so it may be hypothesized that variants in LEPR gene may be associated with NAFLD. In some studies¹¹, polymorphism of LEPR gene (Lys109Arg) is in any way related with NAFLD. Nevertheless, Lu et al¹⁰ suggested that polymorphism of LEPR Pro1019Pro may be an independent predictor of NAFLD. Perhaps, these differences in the influence of the LEPR polymorphisms on hepatic lesions can be explained by the different mutations caused by polymorphism. LEPR Pro1019Pro caused a mutation, it is located in the intracellular domain which is involved in the interactions with Janus-activated kinase (JAK) and signal transducers and activators of transcription (STAT)³⁰. Nevertheless, the cross sectional design of our study showed the lack of association of this polymorphism with liver lesions in NAFLD patients. Therefore, interaction between gene and environmental factors could influence in liver damage or cardiovascular risk factors associated with NAFLD development, such as diabetes mellitus in the following years of these patients. For example, two polymorphisms (Lys109Arg, Gln223Arg) in the extracellular domain of the leptin receptor predicted the conversion to type 2 diabetes patients from high-risk patients (longitudinal studies)³¹.

In conclusion, Lys656Asn polymorphism of LEPR gene is associated with obesity parameters, insulin resistance and glucose levels in patients with NAFLD. In logistic regression analysis, only insulin resistance was associated with portal inflammation, lobular inflammation and steatosis; liver steatosis was related with leptin levels, too. Further studies are needed to further evaluate this exciting area of work, with constant new discoveries^{32,33}.

References

- 1) LUDWIG J, VIGGIANO TR, MCGILL D, OTT BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clinic Proc* 1980; 55: 434-438.
- 2) KRAL JG, SCHAFFNER F, PIERSON RN, WANG J. Body fat topography as an independent predictor of fatty liver. *Metabolism* 1993; 42: 548-551.
- 3) ALLER R, DE LUIS DA, FERNANDEZ L, CALLER F, VELAYOS B, OLCOZ JL, IZAOLA O, GONZALEZ SAGRADO M, CONDE R, GONZALEZ JM. Influence of insulin resistance and adipokines in the grade of steatosis of non-alcoholic fatty liver disease. *Dig Dis Sci* 2008; 53: 1088-1092.
- 4) MARCEAU P, BIRON S, HOULD FS, MARCEAU S, SIMARD S, THUNG SN, KRAL JG. Liver pathology and the metabolic syndrome X in severe obesity. *J Clin Endocrinol Metab* 1999; 84: 1513-1517.
- 5) HUI JM, HODGE A, FARRELL GC, KENCH JG, KRICKETOS A, GEORGE J. Beyond insulin resistance in NASH: TNF-alpha or adiponectin? *Hepatology* 2004; 40: 46-54.
- 6) LEE GH, PROENCA R, MONTEZ JM. Abnormal splicing of leptin receptor in diabetic mice. *Nature* 1996; 379: 632-635.
- 7) CLEMENT K, VAISSE C, LAHLOU N. A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature* 1998; 329: 398-401.
- 8) HEO M, LEIBLE RL, BOYER BB, CHENG WK, KOULU M, KARNOVEN MK, PESONEN U, RISSANEN A, LAAKSO M, UUSITUPA MI, CHAGNON Y, BOUCHARD C, DONOHOU PA, BURNS TL, SHULDINER AR, SILVER K, ANDERSEN RE, PEDERSEN O, ECHWALD S, SØRENSEN TI, BEHN P, PERMUTT MA, JACOBS KB, ELSTON RC, HOFFMAN DJ, ALLISON DB. Pooling analysis of genetic data: The association of leptin receptor (LEPR) polymorphisms with variables related to human adiposity. *Genetics* 2001; 159: 1163-1178.
- 9) KIEFFER TJ, HELLER RS, HABENER JF. Leptin receptors expressed on pancreatic beta-cells. *Biochem Biophys Res Commun* 1996; 224: 522-527.
- 10) LU H, SUN J, SUN L, SHU X, XU Y, XIE D. Polymorphism of human leptin receptor gene is associated with type 2 diabetic patients complicated with NAFLD in China. *J Gastroenterol Hepatol* 2009; 24: 228-232.
- 11) CHEN SH, LI YM, JIANG LL, YU CH. Evaluation of leptin receptor Lys109Arg polymorphism in patients with non-alcoholic fatty liver disease. *Zhonghua Gan Zang Bing Za Zhi* 2006; 6: 453-455.
- 12) BRUNT EM. Non alcoholic steatohepatitis: definition and pathology. *Semin Liver Dis* 2001; 21: 3-16.
- 13) DUART MJ, ARROYO CO, MORENO JL. Validation of an insulin model for the reactions in RIA. *Clin Chem Lab Med* 2002; 40: 1161-1167.
- 14) MATTHEWS DR, HOSKER JP, RUDENSKI AS, NAYLOR BA, TREACHER DF, TURNER RC. Homeostasis model assessment: insulin resistance and beta cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; 28: 412-419.
- 15) WAUTERS M, CONSIDINE R, CHAGNON M, MERTENS I, RANKINEN T, BOUCHARD C, VAN GAAL LF. Leptin levels, leptin receptor gene polymorphisms, and energy metabolism in women. *Obesity Res* 2002; 10: 394-400.
- 16) DE LUIS DA, GONZALEZ M, ALLER R, IZAOLA O, CONDE R. Influence of Lys656Asn polymorphism of leptin receptor gene on insulin resistance in nondiabetic

- obese patients. *J Diabetes Complications* 2008; 22: 199-204.
- 17) DE LUIS DA, GONZALEZ SAGRADO M, ALLER R, IZAOLA O, CONDE R. Influence of Lys565Asn polymorphism of leptin receptor gene on insulin resistance in patients with diabetes mellitus type 2. *Diabetes Res Clin Pract* 2008; 81: e9-e11.
 - 18) BERTI L, KELLERER M, CAPP E, HARING H. Leptin stimulates glucose transport and glycogen synthesis in C2C12 myotubes: evidence for a PI3-kinase mediates effect. *Diabetologia* 1997; 40: 606-609.
 - 19) CEDDIA R, WILLIAM W, CURI R. Comparing effects of leptin and insulin on glucose metabolism in skeletal muscle: evidence for an effect of leptin on glucose uptake and decarboxylation. *Int J Obes* 1999; 23: 75-82.
 - 20) KIEFFER TJ, HABENER JF. The adipoinular axis: effects of leptin on pancreatic beta cells. *Am J Physiol Endocrinol Metab* 2000; 278: E1-E14.
 - 21) MARCHESINI G, BRIZI M, MORSELLI-LABATE A, BIANCHI G, BUGIANESI E, MCCULLOUGH AJ. Association of nonalcoholic fatty liver disease with insulin resistance. *Am J Med* 1999; 107: 450-454.
 - 22) SANYAL AJ, CAMPBELL SARGENT C, MIRSHASHI F, RIZZO WB, CONTOS MJ, STERLING RK, LUKETIC VA. Non alcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 2001; 120: 1183-1192.
 - 23) RASHID M, ROBERTS EA. Nonalcoholic steatohepatitis in children. *J Pediatr Gastroenterol Nutr* 2000; 30: 48-53.
 - 24) CHITTURI S, ABEYGUNASEKERA S, FARRELL GC, HOLMES-WALKER J. NASH and insulin resistance: insulin hypersecretion and specific association with the insulin resistance syndrome. *Hepatology* 2002; 73: 373-379.
 - 25) VENTURI C, ZOPPINI G, ZAMBONI C, MUGGEO M. Insulin sensitivity and hepatic steatosis in obese subjects with normal glucose tolerance. *Nutr Metab Cardiovasc Dis* 2004; 14: 200-204.
 - 26) LECLERCO IA, FARRELL GC, SCHRIEMER R, ROBERTSON GR. Leptin is essential for the hepatic fibrogenic response to chronic liver injury. *J Hepatol* 2002; 37: 206-213.
 - 27) SAHAI A, MALLADI P, PAN X, PAUL R, MELIN-ALDANA H, GREEN RM, WHITINGTON PF. Obese and diabetic db/db mice develop marked liver fibrosis in a model of non-alcoholic steatohepatitis: role of short-form leptin receptors and osteopontin. *Am J Physiol Gastrointest Liver Physiol* 2004; 287: G1035-G1043.
 - 28) EBIHARA K, KUSAKABE T, HIRATA M, MASUZAKI H, MIYANAGA F, KOBAYASHI N, TANAKA T, CHUSHO H, MIYAZAWA T, HAYASHI T, HOSODA K, OGAWA Y, DEPAOLI AM, FUKUSHIMA M, NAKAO K. Efficacy and safety of leptin-replacement therapy and possible mechanisms of leptin actions in patients with generalized lipodystrophy. *J Clin Endocrinol Metab* 2007; 92: 532-541.
 - 29) LEE JH, CHAN JL, SOURLAS E, RAPTOPOULOS V, MANTZOROS CS. Recombinant methionyl human leptin therapy in replacement doses improves insulin resistance and metabolic profile in patients with lipoatrophy and metabolic syndrome induced by the highly active antiretroviral therapy. *J Clin Endocrinol Metab* 2006; 91: 2605-2611.
 - 30) SHINTANI M, IKEGAMI H, YAMATO E, KAWAGUCHI Y, FUJISAWA T, NAKAGAWA Y, HAMADA Y, UEDA H, MIKI T, OGHARA T. A novel micro satellite polymorphism in the human ob gene: a highly polymorphic marker for linkage analysis. *Diabetologia* 1996; 39: 1398-1401.
 - 31) LAKKA T, RANKINEN T, WEISNAGEL SJ, CHAGNON YC, LAKKA HM, UKKOLA O, BOULE N. Leptin and leptin receptor gene polymorphisms and changes in glucose homeostasis in response to regular exercise in nondiabetic individuals. *Diabetes* 2004; 53: 1603-1610.
 - 32) ALLER R, DE LUIS DA, FERNANDEZ L, CALLE H, VELAYOS B, IZAOLA O, GONZALEZ SAGRADO M, CONDE R, GONZALEZ JM. Influence of Ala 54thr polymorphism of fatty acid binding protein 2 on histological alterations and insulin resistance of non alcoholic fatty liver disease. *Eur Rev Med Pharmacol Sci* 2009; 13: 157-162.
 - 33) ALLER R, DE LUIS DA, FERNANDEZ L, CALLER F, VELAYOS B, OLCOZ JL, IZAOLA O, GONZALEZ SAGRADO M, CONDE R, GONZALEZ JM. Influence of insulin resistance and adipokines in the grade of steatosis of non-alcoholic fatty liver disease. *Dig Dis Sci* 2008; 53: 1088-1092.