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# Role of -55CT polymorphism of UCP3 gene on non alcoholic fatty liver disease and insulin resistance in patients with obesity

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Abstract

**Background and aims:** Some studies have pointed to a role of UCP3 in the regulation of biochemical and fat parameters in overweight patients. The aim of our study was to investigate the influence of -55CT polymorphism of UCP3 gene (rs1800849) on histological changes and insulin resistance in patients with non-alcoholic fatty liver disease (NAFLD).

**Material and methods:** A population of 39 patients with NAFLD was recruited in a cross sectional study. The inclusion criterion was the presence of biopsy-proven NAFLD. A biochemical analysis of serum (lipid profile, and adipocytokines) was measured. An anthropometric analysis was assessed, too. Genotype of UCP3 gene -55CT was studied

**Results:** Nine patients (23%) had the genotype 55CC (mutant type group) and 30 patients (77%) 55CT (wild type group). TT genotype was not detected. Insulin levels and HOMA were higher in mutant type group (insulin:  $17.7 \pm 10.9$  mUI/L vs  $11.9 \pm 4.7$  mUI/L;  $p < 0.05$ ) and (HOMA:  $3.2 \pm 1.8$  vs  $4.5 \pm 2.8$ ;  $p < 0.05$ ). Adiponectin levels were lower in mutants type group ( $36.5 \pm 28.1$  ug/ml vs  $21.5 \pm 18.6$  ug/ml;  $p < 0.05$ ). Moderate-severe inflammation and moderate-severe steatosis were more frequent in mutant type group, with higher levels of insulin and lower levels of adiponectin than mild stages.

**Conclusion:** -55CT genotype is associated with high insulin resistance and low adiponectin levels than -55CC genotype. Patients with -55CT genotype have more frequently moderate-severe steatosis and inflammation than -55CC genotype.

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Key words: -55CT polymorphism of UCP3 gene. Biopsy. Insulin resistance. Steatosis. Obesity.

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## EFFECTO DEL POLIMORFISMO -55CT SOBRE EL HÍGADO GRASO Y LA RESISTENCIA A LA INSULINA EN PACIENTES CON SOBREPESO

Resumen

**Introducción y objetivos:** Algunos trabajos han señalado una relación entre el polimorfismo de UCP3 y parámetros bioquímicos y antropométricos en pacientes con sobrepeso. El objetivo de nuestro trabajo es valorar la influencia del polimorfismo -55CT del gen UCP3 (rs1800849) en parámetros histológicos y resistencia a la insulina en paciente con esteatohepatitis no alcohólica (EHNA) y sobrepeso.

**Material y métodos:** Se seleccionó una muestra de 39 pacientes con EHNA. El criterio de inclusión fue la presencia de EHNA comprobado con biopsia hepática. Se realizó una evaluación analítica (lípidos y adipocitoquinas, así como una evaluación antropométrica. Se evaluó el genotipo de UCP3 -55CT.

**Resultados:** Un total de 9 pacientes (23%) presentaron el genotipo mutado 55CC y 30 pacientes (77%) 55CT (genotipo salvaje). El genotipo TT no fue detectado. Los niveles de insulina y de resistencia a la insulina (HOMA) fueron más elevados en los pacientes con genotipo mutado (insulina:  $17,7 \pm 10,9$  mUI/L vs  $11,9 \pm 4,7$  mUI/L;  $p < 0,05$ ) y (HOMA:  $3,2 \pm 1,8$  vs  $4,5 \pm 2,8$ ;  $p < 0,05$ ). Los niveles de adiponectina fueron más bajos en los pacientes con genotipo mutado ( $36,5 \pm 28,1$  ug/ml vs  $21,5 \pm 18,6$  ug/ml;  $p < 0,05$ ). La inflamación moderada-severa y la esteatosis moderada-severa fue más frecuente en el grupo mutado, con niveles mas elevados de insulina y más bajos de adiponectina que en los estadios leves.

**Conclusión:** El genotipo -55CT se asocia a niveles más elevados de insulina y más bajos de adiponectina que el genotipo -55CC. Los pacientes con genotipo -55CT presentan con más frecuencia esteatosis e inflamación en grado moderado severo que los pacientes con genotipo 55CC.

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Palabras clave: -55CT polimorfismo de UCP3. Biopsia. Insulin resistencia. Esteatosis. Obesidad.

## Introduction

Non-alcoholic fat liver disease (NAFLD) is a common liver disease characterized by elevated serum aminotransferase levels, hepatomegaly and accumulation of fat in liver accompanied by inflammation and necrosis resembling alcoholic hepatitis in the absence of heavy alcohol consumption.<sup>1</sup> Although not all patients with NAFLD are obese, obesity is considered the most important risk factor.

In different studies, waist to hip circumference ratio was correlated with degree of steatosis on liver biopsy.<sup>2</sup> Insulin resistance has been associated with fat liver and NAFLD, too.<sup>3</sup> The association with insulin resistance and obesity has also suggested that NAFLD should be considered part of the metabolic syndrome with hyperlipidemia, glucose intolerance, hypertension, and obesity.<sup>4</sup>

Adipose tissue secretes several bioactive proteins, or adipocytokines, that regulate hepatic and peripheral glucose and lipid metabolism. These adipocytokines include leptin, tumor necrosis factor alpha (TNF- $\alpha$ ), resistin, and adiponectin. Recently, Hui et al.<sup>5</sup> 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. Reduced adiponectin level is associated with more extensive liver necroinflammation and may contribute to the development of necroinflammatory forms of NAFLD.<sup>6</sup> However, relationship with genetic factors such as single nucleotide polymorphism remains unknown.

For example, uncoupling protein 3 (UCP3) belongs to a family of mitochondrial transporters that could uncouple the oxidative phosphorylation by increasing the proton leak of the inner mitochondrial membrane.<sup>7</sup> Decreased expression or function of UCP3 could reduce energy expenditure and increase the storage of energy as fat.<sup>8</sup> Some studies have pointed to a role of UCP3 in the regulation of whole body energy homeostasis,<sup>9</sup> diet induced obesity,<sup>10</sup> and regulation of lipids as metabolic substrates.<sup>11</sup>

The C/C genotype of a polymorphism in the UCP3 promoter (-55C->T) (rs1800849) is associated with increased expression of UCP3 mRNA in muscle of Pima Indians.<sup>12</sup> Other authors have reported that the -55 T/T genotype is associated with increased BMI.<sup>13</sup> It was shown that T/T genotype was associated with an atherogenic lipid profile in French Caucasians and with a decreased risk of type 2 diabetes.<sup>14</sup> Recently, a study realized in our country (North Area) has demonstrated an apparently lower risk of obesity in UCP3 -55 C/T carriers.<sup>15</sup>

The aim of our study was to investigate the influence of -55CT polymorphism of UCP3 gene on the histological changes and insulin resistance of NAFLD and adipocytokines levels in overweight patients.

## Subjects and methods

### Subjects

Consecutive 39 overweight Spanish whites born in Castilla-Leon subjects seen between 2004 and 2007 were enrolled for this study. The exclusion criteria was hepatitis B, C, cytomegalovirus, Epstein Barr infections, nonorgan-specific autoantibodies, alcohol consumption, diabetes mellitus, intolerance fasting glucose, medication (blood-pressure lowering medication and statins) and hereditary defects (iron and copper storage diseases and alpha 1-antitrypsin deficiency). These patients were studied in a Nutrition Clinic Unit. The study was approved by the institutional ethics committee. These patients were studied in a Nutrition Clinic Unit and signed an informed consent.

### Liver biopsies

The diagnosis of liver steatosis 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 classification.<sup>16</sup> Steatosis was graded as follows: mild (< 5% of hepatocytes affected); moderate-severe ( $\geq$  5% 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 stage was divided as absent or presence and inflammation (portal and lobulillar) stage was divided as mild or moderate-severe.

### Procedures

Basal glucose, c-reactive protein (CRP), insulin, insulin resistance (HOMA), total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, resistin, leptin, adiponectin, interleukin-6 and TNF- $\alpha$  blood levels were analyzed.

Plasma glucose levels were determined by using an automated glucose oxidase method (Glucose analyser 2, Beckman Instruments, Fullerton, California). Insulin was measured by RIA (RIA Diagnostic Corporation, Los Angeles, CA) with a sensitivity of 0.5 mUI/L (normal range 0.5-30 mUI/L)<sup>17</sup> and the homeostasis model assessment for insulin sensitivity (HOMA) was calculated using these values.<sup>18</sup> CRP was measured by immunoturbimetry (Roche Diagnostcis GmbH, Mannheim, Germany), with a normal range of (0-7 mg/dl) and analytical sensitivity 0.5 mg/dl.

Serum total cholesterol and triglyceride concentrations were determined 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.

Resistin was measured by ELISA (Biovendor Laboratory, Inc., Brno, Czech Republic) with a sensitivity of 0.2 ng/ml with a normal range of 4-12 ng/ml.<sup>19</sup> Leptin was measured by ELISA (Diagnostic Systems Laboratories, Inc., Texas, USA) with a sensitivity of 0.05 ng/ml and a normal range of 10-100 ng/ml.<sup>20</sup> Adiponectin was measured by ELISA (R&D systems, Inc., Minneapolis, USA) with a sensitivity of 0.246 ng/ml and a normal range of 8.65-21.43 ng/ml.<sup>21</sup> Interleukin 6 and TNF alpha were measured by ELISA (R&D systems, Inc., Minneapolis, USA) with a sensitivity of 0.7 pg/ml and 0.5 pg/ml, respectively. Normal values of IL6 was (1.12-12.5 pg/ml) and TNFalpha (0.5-15.6 pg/ml).<sup>22,23</sup>

Weight and body mass index (BMI) were measured. Body weight was measured to an accuracy of 0.5 kg and body mass index computed as body weight/(height<sup>2</sup>).

#### Genotyping of UCP3 gene polymorphism

Oligonucleotide primers and probes were designed with the Beacon Designer 4.0 (Premier Biosoft International®, LA, CA). The polymerase chain reaction (PCR) was carried out with 250 ng of genomic DNA, 0.5 uL of each oligonucleotide primer (primer forward: 5'-GAT CTG GAA CTC ACT CAC CTC-3'; primer reverse: 5'-CTG TTG TCT CTG CTG CTT CT-3'), and 0.25 uL of each probes (wild probe: 5'-Fam-TAT ACA CAC GGG CTG ACC TGA-Tamra-3') and (mutant probe: 5'-Hex-CTT ATA CAC ACA GGC TGA CCT GA- Tamra -3') (Termociclador iCycler IQ (Bio-Rad®), Hercules, CA). DNA was denaturated 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 uL final volume containing 12.5 uL of IQTM Supermix (Bio-Rad®, Hercules, CA) with hot start Taq DNA polymerase.

#### Anthropometric measurements

Body weight was measured to an accuracy of 0.5 kg and body mass index computed as body weight/(height<sup>2</sup>). Waist (narrowest diameter between xiphoid process and iliac crest) and hip (widest diameter over greater trochanters) circumferences to derive waist-to-hip ratio (WHR) were measured, too.

#### Statistical analysis

Sample size was calculated to detect differences over 2 kg of fat mass with 90% power and 5% signifi-

**Table I**  
Changes in anthropometric variables

Parameters	55CC (n = 30)	55CT (n = 9)	p
BMI	29.5 ± 5	28.7 ± 2.5	0.45
Weight (kg)	82.6 ± 12.3	81.9 ± 13	0.34
Fat free mass (kg)	25.3 ± 9	19.5 ± 4.5	0.27
Waist circumference (cm)	97.9 ± 10	94.2 ± 9.5	0.56
Waist to hip ratio	0.9 ± 0.1	0.8 ± 0.1	0.71

(NS) p > 0.05, in each group with basal values.

cance. The statistical analysis was performed for the combined -55CT and -55TT as a mutant group and wild type -55CC as second group. (dominant model). The results were expressed as average ± standard deviation. The distribution of variables was analyzed with Kolmogorov-Smirnov test. Quantitative 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. Qualitative variables were analyzed with the chi-square test, with Yates correction as necessary, and Fisher's test. Correlation analysis was performed with Pearson and Spearman tests. Hardy-Weinberg equilibrium was assessed. A p-value under 0.05 was considered statistically significant.

#### Results

Thirty-nine patients gave informed consent and were enrolled in the study. The mean age was 44.1 ± 9.8 years and the mean BMI 29.2 ± 4.5 with 28 males (71.7%) and 11 females (28.3%). Nine patients (2 females/7 males) (23%) had the genotype 55CT (mutant type group) and 30 patients (6 females/24 males) (77%) 55CC (wild type group). TT genotype was not detected. All subjects were weight stable during the 2 weeks period preceding the study (body weight change, 0.22 ± 0.1 kg).

Table I shows the anthropometric variables in wild and mutant type groups. No differences were detected in fat mass or other anthropometric parameters.

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

Table III shows adipocytokine levels. Patients with 55CC genotype had higher adiponectin levels than mutant type group.

Table IV shows the histological lesions in relation to both genotypes. Patients with mutant type group presented more moderate-severe inflammation (portal and lobulillar) (88.9%) in liver biopsy compared to patient with wild genotype (46.7%). Mutant type group had more moderate-severe steatosis (88.9%) than wild type group (46.7%).

<b>Table II</b> <i>Classical cardiovascular risk factors</i>		
<i>Parameters</i>	<i>55CC (n = 30)</i>	<i>55CT (n = 9)</i>
Glucose (mg/dl)	104.8 ± 28	102.1 ± 12
Total ch. (mg/dl)	218.3 ± 58.4	206.4 ± 33.3
LDL-ch. (mg/dl)	137 ± 52	119.7 ± 24.5
HDL-ch. (mg/dl)	59.4 ± 28.8	56.1 ± 17.5
TG (mg/dl)	125.2 ± 87	107.7 ± 76
Insulin (mUI/L)	11.9 ± 4.7	17.7 ± 10.9*
HOMA	3.2 ± 1.8	4.5 ± 2.8*

Ch.: Cholesterol. TG: Triglycerides  
(\* ) p < 0.05, in each group with basal values.

Patients with moderate-severe steatosis had higher levels of insulin (15.8 ± 7.7 mUI/L vs 10.4 ± 4.7 mUI/L: p < 0.05) and HOMA (4.2 ± 2.2 vs 2.6 ± 1.7: p < 0.05) than patients with mild steatosis. Adiponectin levels were higher in patients with mild steatosis (42.2 ± 24.2 ug/ml vs 26.3 ± 57.0 ug/ml: p < 0.05).

Patients with moderate-severe inflammation had higher levels of insulin (14.9 ± 7.8 mUI/L vs 11.3 ± 5.1 mUI/L: p < 0.05) and HOMA (4.1 ± 2.3 vs 2.9 ± 1.7: p < 0.05) than patients with mild inflammation. Adiponectin levels were higher in patients with mild inflammation (40.5 ± 25.9 ug/ml vs 27.7 ± 26.1 ug/ml: p < 0.05).

## Discussion

The ubiquitous expression of UCP2, the expression of UCP3 in skeletal muscle, and their homology with UCP1 made UCP3 attractive targets for studies on obesity patients and its relation with biochemical and anthropometric parameters in NAFLD patients.<sup>24</sup> UCP3 is involved in thermogenesis through the uncoupling of oxidative phosphorylation in skeletal muscle.

The C/C genotype of the polymorphism in the UCP3 promoter (-55C-> T) is associated with obesity. The frequency of C to T variant at -55 of the UCP3 gen was evaluated, frequency of the T allele was 26% among obese draftees and 26.9% in the control group.<sup>25</sup> Our group had similar prevalence, without differences in

<b>Table III</b> <i>Circulating adipocytokines levels</i>		
<i>Parameters</i>	<i>55CC (n = 30)</i>	<i>55CT (n = 9)</i>
TNF (pg/mL)	5.4 ± 3.8	4.8 ± 3.3
Leptin (ng/mL)	35.1 ± 35.5	40.1 ± 29.2
IL-6 (ng/mL)	2.2 ± 3.2	2.1 ± 3.5
Adiponectin (ug/mL)	36.5 ± 28.1	21.5 ± 18.6*
Resistin (ug/mL)	2.7 ± 1.2	3.1 ± 0.7

(\* ) p < 0.05.

<b>Table IV</b> <i>Histological parameters</i>		
<i>Parameters</i>	<i>55CC (n = 30)</i>	<i>55CT (n = 9)</i>
<i>Steatosis</i>		
Mild	16 (53.3%)	1 (11.1%)*
Moderate-Severe	14 (46.7%)	8 (88.9%)*
<i>Portal and lobulillar inflammation</i>		
Mild	16 (53.3%)	1 (11.1%)*
Moderate-severe	14 (46.7%)	8 (88.9%)*
<i>Fibrosis</i>		
Mild	24 (80.0%)	8 (88.9%)
Moderate-severe	6 (20.0%)	1 (11.1%)

Chi square test, differences in each stage (\* ) p < 0.05.  
(%) frequencies in each genotype.

weight or other anthropometric parameters between wild and mutant type groups. However, Liu et al.<sup>26</sup> found statistically association and linkage between -55CT and BMI, and subjects carrying the T allele had an average of 3.5% lower BMI than those without it. In the study by Cassell et al.<sup>27</sup> the waist to hip ratio was higher in females carrying the UCP3 gene -55CT polymorphism, but BMI was no different in both groups. However, other study has demonstrated a lower risk of obesity in UCP3 -55 C/T carriers.<sup>15</sup> These contradictories data has not a clear explanation, perhaps other unknown interactions between genotype and environment could modulate the results of UCP3's studies. A second hypothesis to explain the different frequency of the minor allele UCP3-55T and the lack of association with obesity, a possibility is that the genotyping methods did not produce accurate results and a second method to assess accuracy has not been realized.

In our population, adiponectin was higher in wild type group with better insulin and HOMA levels than mutant type group; this is a novel result in the literature without a clear explanation. Perhaps the presence of mutant allele of UCP could produce a more proinflammatory state, for instance in skeletal muscle and adipose tissue may modified the production of cytokines. For example, Meirhaeghe et al.<sup>14</sup> have detected that TT genotype had a worse lipid profile than subjects bearing wild or heterozygous genotypes.

The other novel finding of our study is the relationship of UCP3 -55CT polymorphism with moderate-severe inflammation and steatosis on liver biopsy. Perhaps, the explanation of this relation is the high levels of insulin and insulin resistance in mutant type group patients. Marchesini et al.<sup>28</sup> demonstrated a closely correlation between insulin resistance (HOMA) and NAFLD, too. Other authors have been detected this relation using the clamp technique<sup>29-31</sup> with results supporting our conclusions.

In our study, adiponectin may protect against inflammation and steatosis also through its anti-inflammatory action. Adiponectin inhibits liver TNF alpha expression

and also inhibits expression of several cytokines in hepatic stellate cells.<sup>32</sup> Serum adiponectin levels are reduced with insulin resistance,<sup>33</sup> this relation was detected in our study, patients with moderate-severe steatosis and inflammation had higher levels of insulin, HOMA and lower levels of adiponectin than patients with mild steatosis and inflammation.

In conclusion, -55CT genotype is associated with high insulin resistance and low adiponectin levels than -55CC genotype. Patients with -55CT genotype have more frequently moderate-severe steatosis and inflammation than -55CC genotype. Adiponectin and insulin resistance may be the biochemical nexus.

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