

Original

## Polymorphism Trp64Arg of beta 3 adrenoreceptor gene: allelic frequencies and influence on insulin resistance in a multicenter study of Castilla-León

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### Abstract

**Background and objective:** The genetic variant (Trp64Arg) is a missense mutation located within the beta3 adrenoreceptor (Beta3AR). The aim of our study was to investigate the influence of Trp64Arg polymorphism in the Beta3AR gene on insulin resistance in obese patients and the allelic distribution of this polymorphism in a geographic area of Spain.

**Design:** A population of 264 obese patients was analyzed. A bioimpedance, blood pressure, an assessment of nutritional intake, and biochemical parameters were measured. The beta 3 adrenoreceptor gene polymorphism (Trp64Arg) was genotyped.

**Results:** Two hundred and twenty six patients (77 males/149 females) (85.6%) had the genotype Trp64/Trp64 (wild type group) with an average age of 41.12 ± 13.1 years and 38 patients (16 males/22 females) Trp64/Arg64 (14.4%) (mutant type group) with an average age of 40.5 ± 12.7 years. High frequencies of Arg64 allele were observed in Salamanca and Valladolid. In the mutant type group, HOMA (3.75 ± 2.77 vs 5.27 ± 5.4; p < 0.05) was higher than wild type group.

**Conclusion:** The finding of this study is the association of the Trp64/Arg64 Beta3AR with higher levels of HOMA. Frequencies of this polymorphism are different among geographic areas.

(Nutr Hosp. 2010;25:299-303)

DOI:10.3305/nh.2010.25.2.4599

Key words: Castilla-León. Obesity. Trp64Arg beta 3 adrenoreceptor.

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Recibido: 17-XI-2009.  
Aceptado: 22-XI-2009.

### POLIMORFISMO TRP64ARG DEL GEN RECEPTOR BETA 3: FRECUENCIA ALÉLICA E INFLUENCIA EN LA RESISTENCIA A LA INSULINA EN UN ESTUDIO MULTICÉNTRICO DE CASTILLA Y LEÓN

#### Resumen

**Introducción y objetivos:** La variante genética (Trp64Arg) es una mutación localizada en el adrenoreceptor Beta 3 (Beta3AR). El objetivo de nuestro trabajo es evaluar la influencia de el polimorfismo Trp64Arg del gen de Beta3AR sobre la resistencia a la insulina en pacientes obesos, así como la distribución alélica de este polimorfismo en un área geográfica de España. **Diseño:** Una muestra de 264 pacientes obesos fue analizada. Se realizó una bioimpedancia, evaluación nutricional y análisis bioquímico. Se genotiparon a los pacientes en función del polimorfismo Trp64Arg del gen adrenoreceptor-beta 3.

**Resultados:** Un total de 227 pacientes (77 varones/149 mujeres) (85,6%) presentaron el genotipo Trp64/Trp64 (grupo genotipo salvaje), con una media de edad de 41,12 ± 13,1 años y un total de 38 pacientes (16 varones/22 mujeres) Trp64/Arg64 (14,4%) (grupo genotipo mutante) con una edad media de 40,5 ± 12,7 años. Se detectó una alta frecuencia alélica (Arg64) en las áreas de Salamanca y Valladolid. En el grupo mutante, la resistencia a la insulina (HOMA) (3,75 ± 2,77 vs 5,27 ± 5,4; p < 0,05) fue más alta que en grupo con genotipo salvaje.

**Conclusion:** Existe una asociación entre el genotipo mutante del polimorfismo Trp64/Arg64. Las frecuencias alélicas del polimorfismo son diferentes en función de la áreas de salud.

(Nutr Hosp. 2010;25:299-303)

DOI:10.3305/nh.2010.25.2.4599

Palabras clave: Castilla y León. Obesidad. Trp64Arg beta 3 adrenoreceptor.

## Introduction

Obesity has multiple causes, and it is determined by the interaction between genetic and environmental factors.<sup>1</sup> Especially, gene defects showing no or only minor effect when expressed alone might influence on the phenotype. Genetic background of these patients could influence in follow up and outcomes.<sup>2,3</sup>

A genetic variant is the tryptophan-to-arginine (Trp64Arg) missense mutation in the beta3 adrenoreceptor (Beta3AR). Beta3-AR is the principle mediator of catecholamine-stimulated thermogenesis and lipolysis.<sup>4</sup> Trp64Arg variant in this receptor has been reported to be associated with increased body weight and insulin resistance.<sup>5</sup> Since the publication of this polymorphism, numerous studies have been conducted to investigate the relationship of this common variant with various phenotypes of obesity. However, such findings have not been consistent among the studies subsequently undertaken raising questions about the significance of a possible relationship between the Trp64Arg polymorphism and these clinical features. Adipose tissue is considered an active secretory organ, sending out and responding to signals that modulate appetite, insulin sensitivity, energy expenditure, and inflammation.

The aim of our study was to investigate the influence of Trp64Arg polymorphism in the Beta3 adrenoreceptor gene on obesity anthropometric parameters and insulin resistance in obese patients and the allelic distribution of this polymorphism in a geographic area of Spain (Castilla-León).

## Subjects and methods

### Subjects

A population of 264 obesity (body mass index > 30) patients was analyzed in a prospective way (research protocol accepted by ethical committee). These patients were studied in the Nutrition Clinic Units and signed an informed consent. The recruitment of subjects was a non probabilistic method of sampling among patients send from Primary Care Physicians with obesity to each Unit of Nutrition of each Health Area of Castilla Leon in the Northwest of Spain (Avila (n = 156,535 habitants), Burgos (n = 350,122 habitants), Leon (n = 473,407 habitants), Palencia (n = 165,740 habitants), Salamanca (n = 335,407 habitants), Segovia (n = 141,750 habitants), Soria (n = 90,521 habitants), Valladolid (n = 507,297 habitants), Zamora (n = 182,710 habitants)). Exclusion criteria included history of cardiovascular disease or stroke during the previous 36 months, total cholesterol > 300 mg/dl, triglycerides > 400 mg/dl, blood pressure > 140/90 mmHg, fasting plasma glucose > 110 mg/dl, as well as the use of sulfonylurea, thiazolidinedionas, insulin, glucocorticoids, antineoplastic agents, angiotensin

receptor blocker, angiotensin converting enzyme inhibitors, psychoactive medications, drinking and/or smoking habit.

### Procedure

All patients with a 2 weeks weight-stabilization period before recruitment were enrolled. Weight, blood pressure, basal glucose, c-reactive protein (CRP), insulin, total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides blood levels were measured. Genotype of beta 3 adrenoreceptor gene polymorphism was studied.

### Genotyping of Beta3 adrenoreceptor 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'-CAA CCT GCT GGT CAT CGT-3'; primer reverse: 5'-AGG TCG GCT GCG GC-3'), and 0.25 uL of each probes (wild probe: 5'-Fam-CCA TCG CCT GGA CTC CG-BHQ-1-3') and (mutant probe: 5'-Hex-CAT CGC CCG GAC TCC G- BHQ-1-3') in a 25 uL final volume (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.

### Assays

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.

Plasma glucose levels were determined by using an automated glucose oxidase method (Glucose analyser 2, Beckman Instruments, Fullerton, California). Insulin was measured by enzymatic colorimetry (Insulin, WAKO Pure-Chemical Industries, Osaka, Japan) and the homeostasis model assessment for insulin sensitivity (HOMA) was calculated using these values.<sup>6</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.

### 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. Tetrapolar body electrical bioimpedance was used to determine body composition.<sup>7</sup> An electric current of 0.8 mA and 50 kHz was produced by a calibrated signal generator (Biodynamics Model 310e, Seattle, WA, USA) and applied to the skin using adhesive electrodes placed on right-side limbs. Resistance and reactance were used to calculate total body water, fat and fat-free mass.

Blood pressure was measured twice after a 10 minutes rest with a random zero mercury sphygmomanometer, and averaged.

### Dietary intake and habits

Patients received prospective serial assessment of nutritional intake with 3 days written food records. All enrolled subjects received instruction to record their daily dietary intake for three days including a weekend day. Handling of the dietary data was by means of a personal computer equipped with personal software, incorporating use of food scales and models to enhance portion size accuracy. Records were reviewed by a registered dietitian and analyzed with a computer-based data evaluation system. National composition food tables were used as reference.<sup>8</sup> Regular aerobic physical activity (walking was allowed, no other exercises) was maintained during the period of study (2-3 hours per week).

### Statistical analysis

Sample size was calculated to detect differences over 1 point of HOMA with 90% power and 5% significance.

The results were expressed as average  $\pm$  standard deviation. The distribution of variables was analyzed with Kolmogorov-Smirnov test. Quantitative variables with normal distribution were analyzed with a two-tailed, Student's-t test. Non-parametric variables were analyzed with the U-Mann Whitney test. Qualitative variables were analyzed with the chi-square test, with Yates correction as necessary, and Fisher's test. Correlation analysis was realized with Pearson test and Spearman test. The statistical analysis was performed for the combined *Trp64/Arg64* and *Arg64/Arg64* as a mutant group and type *Trp64/Trp64* as wild group with a dominant model. Hardy Weinberger equilibrium was assessed. A p-value under 0.05 was considered statistically significant.

### Results

Two hundred and sixty four patients gave informed consent and were enrolled in the study. The mean age was  $41.1 \pm 13.1$  years and the mean BMI  $36.5 \pm 5.9$ , with 94 males (35.6%) and 170 females (74.4%).

Two hundred and twenty six patients (77 males/149 females) (85.6%) had the genotype *Trp64/Trp64* (wild type group) with an average age of  $41.12 \pm 13.1$  years and 38 patients (16 males/22 females) *Trp64/Arg64* (14.4%) (mutant type group) with an average age of  $40.5 \pm 12.7$  years.

Table I shows distribution of genotypes and allelic frequencies in different Health Areas. High frequencies of Arg64 allele were observed in Salamanca and Valladolid.

Table II shows the anthropometric variables. No statistical differences were detected between genotypes.

Table III shows the differences in cardiovascular risk factors. In mutant type group, HOMA was higher than wild type group.

Table IV shows nutritional intake with 3 days written food records. No statistical differences were detected in calorie, carbohydrate, fat, and protein intakes.

**Table I**  
Genotype and allelic distribution in different health areas

Areas	<i>Trp64/Trp64</i>	<i>Trp64/Arg64</i>	<i>Trp64</i>	<i>Arg64</i>
Avila (n = 12)	86.4%	13.6%	0.93	0.07
Burgos (n = 35)	91.4%	8.6%	0.95	0.05
Leon (n = 61)	86.3%	13.7%	0.94	0.06
Palencia (n = 12)	91.7%	8.3%	0.95	0.05
Salamanca (n = 7)	71.4%*	28.6%*	0.85+	0.15
Segovia (n = 21)	100%	0%	1.00	0.00
Soria (n = 18)	88.9%	11.1%	0.94	0.06
Valladolid (n = 64)	77.7%*	22.3%*	0.88+	0.12
Zamora (n = 24)	83.3%	16.7%	0.92	0.08

(\*) P < 0.05, statistical differences in the same genotype among different Health Areas.

(+) P < 0.05, statistical differences in the same allele among different Health Areas.

**Table II**  
*Differences in anthropometric variables*

Characteristics	Trp64/Trp64 n = 226	Trp64/Arg64 n = 38
BMI	36.6 ± 5.6	36.1 ± 7.4
Weight (kg)	98.3 ± 19.2	96.8 ± 20.4
Fat free mass (kg)	56.3 ± 13.1	54.2 ± 13.7
Fat mass (kg)	39.6 ± 12.4	40.5 ± 16.4
Waist circumference(cm)	111.8 ± 14.3	110.6 ± 15.2
Waist to hip ratio	0.93 ± 0.09	0.92 ± 0.07
Systolic BP (mmHg)	126.9 ± 16.6	130.1 ± 18.1
Diastolic BP (mmHg)	79.2 ± 12.1	80.9 ± 7.8

BMI: body mass index. BP: Blood pressure. No statistical differences between genotypes.

## Discussion

In the mutant group of beta3 adrenoreceptor gene (*Trp64/Arg64*) patients have higher HOMA than wild type group. Two Health Areas (Salamanca and Valladolid) have a high frequency of this polymorphism compared with others of Castilla León.

Our present finding that the global frequency of the Arg64 allele in Castilla-León was 14.4% in obese patients agrees with previous reports.<sup>9-11</sup> The results of a meta-analysis assessing quantitative phenotypes in relation to a genetic polymorphism, and the result support the association of Trp64Arg polymorphism with BMI across diverse populations.<sup>12</sup> However, other meta-analysis did not find evidence of this association,<sup>13</sup> as our multi center study shows.

Other controversial area is the relationship of this polymorphism and glucose metabolism disorders. Mice with knockout of the Beta 3 adrenoreceptor gene showed marked reductions in lipolysis stimulated by Beta 3 agonists,<sup>14</sup> and omental adipocyte Beta 3 adrenoreceptor sensitivity was related to waist to hip ratio and insulin resistance.<sup>15</sup> Perhaps, defects in beta 3

**Table III**  
*Classical cardio vascular risk factors*

Characteristics	Trp64/Trp64 n = 226	Trp64/Arg64 n = 38
Glucose (mg/dl)	98.2 ± 24.7	102.9 ± 34.1
Total ch. (mg/dl)	188.0 ± 37.2	185.6 ± 38.6
LDL-ch. (mg/dl)	107.9 ± 42.4	102.8 ± 42.8
HDL-ch. (mg/dl)	66.1 ± 36.2	63.4 ± 32.8
TG (mg/dl)	122.6 ± 67.4	116.5 ± 55.2
Insulin (mUI/L)	16.3 ± 13.3	21.6 ± 22.9
HOMA	3.75 ± 2.77	5.27 ± 5.4*
CRP (mg/dl)	4.90 ± 7.30	4.86 ± 10.4

Chol: Cholesterol. TG: Triglycerides. HOMA: homeostasis model assessment. (\*p < 0.05) statistical differences between genotypes.

**Table IV**  
*Dietary intake*

Characteristics	Trp64/Trp64	Trp64/Arg64
Energy (kcal/day)	2023 ± 817	1979 ± 649
CH (g/day)	200.6 ± 64.4	197.1 ± 75.3
Fat (g/day)	91.5 ± 51.5	79.3 ± 33.1
Protein (g/day)	94.8 ± 40.3	87.6 ± 31.8

No statistical differences between groups. CH: Carbohydrate.

adrenoreceptor signal transduction, binding, or regulatory mechanism may result in a diminished lipolytic response in visceral adipose tissue, aggravating insulin resistance. However, other authors have detected an inverse relation between visceral obesity in Trp64Arg mutation patients and serum triglyceride.<sup>16</sup> Our data showed higher insulin resistance in mutant type group than wild type group. This relationship with a lack of association with body mass index has been detected in a middle-aged white population from Denmark,<sup>17</sup> too. In this study the Trp64Arg polymorphism was not associated with obesity. However the Arg allele was associated with an increased insulin resistance estimated by HOMA. Bracale et al have demonstrated similar results in a population from Italy.<sup>18</sup>

The mechanism through which the Arg64 variant alters insulin sensitivity could be explained by adipocytokine actions. Resistin and adiponectin appear to be important in regulating insulin sensitivity.<sup>19</sup> Perhaps, these unclear results in the literature<sup>20</sup> may partially be explained by differences in ethnic background, baseline BMI, gender distribution, previous weight loss, experimental design (early stage or late stage type 2 diabetes mellitus) and basal adipocytokines levels of participants. Therefore, interaction between gene and ambient could explain these differences with bias in previous studies. Perhaps these differences could be explained by a dietary bias. Gene-environment interaction studies would require composition analysis of the diet to determine whether dietary components could be responsible for the lipid differences. In our study dietary intake did not show statistical differences between groups, in this way our dates have controlled by dietary intake and previous discrepancies could be explain by this uncontrolled factor (dietary intake). Geographic area of the populations could be other factor to take account.

Geographic variations of prevalence have been described in other single nucleotide polymorphism such as Ala54Thr of Fatty acid binding protein.<sup>20</sup> Other results are consistent with the idea that climate has been an important selective pressure acting on candidate genes for common metabolic disorders such as LEPR polymorphisms.<sup>21</sup> In one study with hypertensive obese patients from China, the allele frequency of 64Arg was 0.18.<sup>22</sup> Other study from Japan showed an allele frequency of Arg64 of 18%.<sup>23</sup> Both studies

showed higher frequencies than our global area (Castilla Leon) but similar that two Health Areas (Val-ladolid and Salamanca).

This association could have therapeutically implications. Interventional studies with this polymorphism are limited.<sup>24</sup> This study showed that the beneficial effect of mild weight reduction by a low caloric diet and exercise program is the greatest in subjects with normal homozygotes beta 3 adrenoreceptor gene. In other study, after obese children were subjected to a low-fat diet for 3 months, the increased range in body weight in obese children without the mutation was less than that in obese with mutation and the control group.<sup>25</sup>

The finding of this study is the association of the Trp64/Arg64 Beta3AR with higher levels of HOMA. Frequencies of this polymorphism are different among geographic areas.

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