

## ***LEPR* p.Q223R, $\beta$ 3-*AR* p.W64R and *LEP* c.-2548G>A gene variants in obese Brazilian subjects**

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**ABSTRACT.** Obesity is due to the combined effects of genes, environment, lifestyle, and the interactions of these factors. The adrenergic receptor  $\beta$ 3 ( *$\beta$ 3-AR*), leptin (*LEP*) and leptin receptor (*LEPR*) genes have been intensively evaluated in the search of variants that could be related to obesity and its cardiometabolic complications. The results of most of these studies have been controversial. In the present study, we investigated the relationship of the  *$\beta$ 3-AR* p.W64R, *LEP* c.-2548G>A and *LEPR* p.Q223R gene variants with body mass index (BMI), in Brazilian subjects of different genetic backgrounds and ethnic origins. Two hundred obese patients (60 males, 140 females, BMI  $\geq$  30 kg/m<sup>2</sup>) were screened and compared to 150 lean healthy subjects (63 males, 87 females, BMI  $\leq$  24 kg/m<sup>2</sup>). Genomic DNA was extracted and amplified by polymerase chain reaction. Polymerase chain reaction products were digested with specific restriction enzymes and separated by electrophore-

sis. There was no significant difference in the genotype frequency of the  $\beta 3$ -AR p.W64R and the *LEP* c.-2548G>A polymorphisms, between lean and obese subjects. However, the genotype and allele frequencies of the *LEPR* p.Q223R variant were significantly different between the normal weight and obese groups. Haplotype analysis has shown an association between the G/G allelic combination of c.-2548G>A *LEP* and c.668A>G *LEPR*, in obese subjects. Our results suggest that genetic variability in the leptin receptor is associated with body weight regulation, the *LEPR* p.Q223R variant being related to BMI increase. The haplotype combination of *LEP* c.-2548G>A and *LEPR* p.Q223R variants was related to a 58% increase in obesity risk.

**Key words:** Genetic polymorphism, Obesity,  $\beta 3$ -adrenergic receptor, Leptin, Leptin receptor

## INTRODUCTION

Weight excess affects not only westernized industrialized societies, but developing countries as well (Hedley et al., 2004; IBGE, 2004). A national survey conducted in 2002-2003 with 95.5 million Brazilian adults over the age of 20 years revealed that 38.8 million (40.6%) of the individuals examined from this country were overweight, of whom 10.5 million were obese (IBGE, 2004).

The increasing prevalence of obesity throughout the world has triggered a search for candidate genes involved in the disease. Most of the obesity-predisposing genes encode the molecular components of the physiological systems regulating energy balance (Barsh and Schwartz, 2002). The adrenergic receptor  $\beta 3$  ( $\beta 3$ -AR), leptin (*LEP*) and leptin receptor (*LEPR*) genes have been evaluated in the search for variants that could be potentially related to the pathophysiology of obesity and its complications.

The  $\beta 3$ -AR is expressed especially in visceral fat, and has a central role in the mechanisms leading to increased lipolysis in response to catecholamines (Miyaki et al., 2005). The p.W64R  $\beta 3$ -AR polymorphism decreases receptor sensitivity (Clement et al., 1995; Miyaki et al., 2005), and may be associated with weight gain.

Rare obesity syndromes are associated with mutations of the leptin gene in humans (Montague et al., 1997; Strobel et al., 1998). One polymorphism identified in the 5' untranslated region - the c.-2548G>A - has been evaluated in different populations, but the associations of that variant with obesity have been inconclusive (Li et al., 1999; Mammès et al., 2000; Poitou et al., 2005; Wang et al., 2006). Unlike with the *LEP* gene, approximately 10 polymorphic sites have already been reported in the *LEPR* gene (Heo et al., 2001). However, the association of those variants with obesity has been contradictory (Heo et al., 2001, 2002; Paracchini et al., 2005).

To address the question of the conflicting nature of these studies, and the necessity to conduct additional analyses, particularly in economically emerging populations, we proposed to investigate the possible relationships of the  $\beta 3$ -AR, *LEP* and *LEPR* gene variants with body mass index (BMI), in Brazilian subjects of different ethnic origins.

## MATERIAL AND METHODS

### Subjects

Our sample included 150 lean subjects (63 men and 87 women) recruited among the healthy volunteer blood donors of the blood bank of the Rio de Janeiro State University Hospital, with ages ranging from 19 to 61 years ( $32.0 \pm 9.7$  years), and 200 obese subjects (60 men and 140 women) with ages ranging from 18 to 71 years ( $42.3 \pm 12.1$  years), selected from the University Hypertension Clinic. All participants lived in the urban area of Rio de Janeiro, in the southeast of Brazil. There were important ethnic differences in the population studied because it was composed of European-Caucasians, mulattoes (people of mixed (African and Caucasian) ancestry) and autochthonous Amerindians. According to the World Health Organization classification, 49.5% of our patients were classified as grade 1 obese, 25.5% as grade 2 obese, and 25% as grade 3 obese. The BMI of lean subjects was lower than  $24 \text{ kg/m}^2$  and stable for at least 3 years. The BMI values of the obese patients ranged from 30 to  $70 \text{ kg/m}^2$  (mean BMI =  $38.49 \text{ kg/m}^2$ ). The research protocol was approved by the Ethics Committee of the Rio de Janeiro State University Hospital, and written informed consent was obtained from all the patients.

### Molecular analysis

Genomic DNA was obtained from peripheral blood leukocytes using the salting-out method (Miller et al., 1988). Genotyping of the three polymorphisms [ $\beta 3$ -AR c.189T>C (p.W64R), *LEP* c.-2548G>A, *LEPR* c.668A>G (p.Q223R)] was carried out by use of the polymerase chain reaction-restriction fragment length polymorphism assay with previously described primer pairs (Clement et al., 1995; Gotoda et al., 1997; Mammès et al., 2000). Separate polymerase chain reactions were performed in total volumes of 25  $\mu\text{L}$  using 100 ng DNA, 1X PCR buffer, 2.0 mM  $\text{MgCl}_2$ , 0.2-0.25 mM of each dNTP, 2.5-5 pmol of each primer, and 0.5-1.5 U Taq DNA polymerase. DNA was denatured for 5 min at  $95^\circ\text{C}$ , and amplified for 40 cycles with annealing temperatures of  $66^\circ\text{C}$  for  $\beta 3$ -AR p.W64R,  $51^\circ\text{C}$  for *LEP* c.-2548G>A and  $52^\circ\text{C}$  for *LEPR* p.Q223R, and 10  $\mu\text{L}$  of the polymerase chain reaction products was digested with 2.5 U of the restriction endonucleases *Bst*NI ( $\beta 3$ -AR p.W64R), *Hha*I (*LEP* c.-2548G>A) and *Msp*I (*LEPR* p.Q223R) according to the manufacturer's recommendations (New England Biolabs, Inc.), and analyzed by electrophoresis on 8% polyacrylamide gels stained with silver nitrate (Santos et al., 1993).

### Statistical analysis

Allele frequencies were determined by gene-counting. The chi-square test was used to verify the significant association between obesity and genotypes. Odds ratios (OR) were used to express the risk of obesity associated with a particular genotype. Adjusted OR were estimated using stepwise logistic regression to infer the potential imbalance of sex and age. The significance level adopted for the statistical tests was 0.05. The results are presented as means  $\pm$  standard deviation. These statistical analyses were performed by SPSS for Windows software, version 14.0.

Additionally, linkage disequilibrium, association and haplotype analyses were performed using the Arlequin, GDA, H+Plus and Chaplin programs.

## RESULTS

The genotype and allele frequencies and their associations with BMI are shown in Table 1. There were no differences between obese and lean subjects concerning genotype frequency of the  $\beta 3$ -AR p.W64R (OR = 0.84; 95%CI = 0.47-1.49) and *LEP* c.-2548G>A (OR = 0.85; 95%CI = 0.55-1.30) polymorphisms. There was a significant difference in *LEPR* p.Q223R polymorphism when comparing obese and lean subjects (OR = 1.65; 95%CI = 1.04-2.60). The association of the *LEPR* p.Q223R polymorphism with obesity was related to the co-dominant and dominant model, but not with the recessive model (Table 1). There was still an association between *LEPR* p.Q223R polymorphism and obesity after adjusting for sex and age (OR = 1.79; 95%CI = 1.11-2.90). There were no differences concerning the frequency of the polymorphisms and the grade of obesity.

**Table 1.** Genotype and allele frequencies of the  $\beta 3$ -AR p.W64R, *LEP* c.-2548G>A, *LEPR* p.Q223R polymorphisms in lean (BMI  $\leq$  24 kg/m<sup>2</sup>), and obese (BMI  $\geq$  30 kg/m<sup>2</sup>) Brazilian subjects.

Genotypes	Polymorphisms								
	$\beta 3$ -AR (p.W64R)			<i>LEP</i> (c.-2548G>A)			<i>LEPR</i> (p.Q223R)		
	Obese N = 200 (%)	Lean N = 150 (%)	$\chi^2/P$	Obese N = 200 (%)	Lean N = 150 (%)	$\chi^2/P$	Obese N = 200 (%)	Lean N = 150 (%)	$\chi^2/P$
<b>Co-dominant model</b>									
1/1	170 (85%)	124 (82%)	0.57/0.75	108 (54%)	75 (50%)	0.59/0.74	53 (26%)	56 (38%)	5.95/0.05
1/2	28 (14%)	25 (17%)		76 (38%)	61 (41%)		120 (60%)	71 (47%)	
2/2	2 (1%)	1 (0.7%)		16 (8%)	14 (9%)		27 (14%)	23 (15%)	
<b>Dominant model</b>									
1/1	170 (85%)	124 (83%)	0.35/0.55	108 (54%)	75 (50%)	0.55/0.46	53 (26.50)	56 (37%)	4.69/0.03
1/2 + 2/2	30 (15%)	26 (17%)		92 (46%)	75 (50%)		147 (73%)	94 (63%)	
<b>Recessive model</b>									
1/1 + 1/2	198 (99%)	149 (99%)	0.11/0.74	184 (92%)	136 (90%)	0.19/0.66	173 (87%)	127 (85%)	0.24/0.63
2/2	2 (1%)	1 (1%)		16 (8%)	14 (9%)		27 (14%)	23 (15%)	
<b>Alleles</b>									
1	91%	92%	0.22/0.64	73%	70%	0.60/0.44	57%	61%	1.43/0.23
2	9%	8%		27%	30%		43%	39%	

1 = wild-type allele; 2 = polymorphic allele;  $\beta 3$ -AR = adrenergic receptor  $\beta 3$ ; *LEP* = leptin; *LEPR* = leptin receptor.

Linkage disequilibrium between the polymorphisms c.-2548G>A (of *LEP* gene) and c.668A>G (of *LEPR* gene) was observed in the obesity group ( $\chi^2_{(1)} = 7.1521$ ;

P = 0.0074) but not in the lean group ( $\chi^2_{(1)} = 0.7947$ ; P = 0.3726). Haplotype analysis showed an association between the G/G allelic combination of c.-2548G>A (*LEP* gene) and c.668A>G (*LEPR* gene), respectively, in obese subjects (OR = 1.58; 95%CI = 1.04-2.39) (Table 2).

**Table 2.** Results of haplotype analysis showing that the allelic association of *LEP* (c.-2548G>A) with *LEPR* (c.668A>G) variants increases significantly the risk of obesity.

Haplotype		Control frequency	Case frequency	Odds ratio	Confidence interval	P value
<i>LEP</i> (c.-2548G>A)	<i>LEPR</i> (c.668A>G)					
G	A	0.41761	0.36863	1.00	-	-
G	G	0.28572	0.36137	1.58	1.04 - 2.39	0.032
A	A	0.19239	0.19637	1.29	0.81 - 2.06	0.291
A	G	0.10428	0.07363	0.74	0.33 - 1.64	0.461

LEP = leptin; LEPR = leptin receptor.

## DISCUSSION

The p.Q223R polymorphism of the *LEPR* gene, but neither the p.W64R of the  $\beta$ -*AR* gene nor the c.-2548G>A of the *LEP* gene, was associated with obesity in a sample of multiethnic Brazilians. A haplotype association between *LEP* and *LEPR* variants and BMI was shown in these subjects.

The frequency of the C allele of c.189T>C polymorphism in the  $\beta$ -*AR* gene was within the range observed in Caucasian and Asian populations (Gagnon et al., 1996; O'Dell et al., 1998; Lowe Jr. et al., 2001; Kim et al., 2003; Hao et al., 2004; Ramis et al., 2004), including the results from a European-derived population from southern Brazil (Mattevi et al., 2006). The prevalence of the C allele has been reported to be substantially higher in more primitive populations such as the Eskimos from Alaska (Biery et al., 1997) and the Pima Indians (Walston et al., 1995), in Mexican Americans (Mitchell et al., 1998), and in urban populations of Japan (Arashiro et al., 2003) and southern Korea (Kim et al., 2003).

Several studies have shown an association between the c.189T>C variant in the  $\beta$ -*AR* and obesity- and diabetes-related phenotypes (O'Dell et al., 1998; Allison et al., 1998; Oizumi et al., 2001). This association was first documented in Pima Indians and afterwards in different ethnic groups (Walston et al., 1995; Widen et al., 1995; Mitchell et al., 1998; Hao et al., 2004; Miyaki et al., 2005). Our results show no evidence for a relationship between the genotype and allele frequencies of the c.189T>C variant and BMI, coinciding with other reports (Gagnon et al., 1996; Nagase et al., 1997; Garenc et al., 2001; Lowe Jr. et al., 2001; Rawson et al., 2002; Mattevi et al., 2006). The results of three meta-analyses on linkage and association of the c.189T>C variant with BMI have also been conflicting (Allison et al., 1998; Fujisawa et al., 1998; Kurokawa et al., 2001). In one review (Fujisawa et al., 1998) that selected 23 studies and included 7399 subjects from different parts of the world, no association of the p.W64R polymorphism with obese phenotypes was reported. The other two meta-analyses (Allison et al., 1998; Kurokawa et al., 2001), one including 9000 subjects of distinct populations from Finland, France, Japan, Sweden, USA, Australia, and Denmark, and another including 6582 Japanese, concluded that there was a significant effect of this polymorphism on BMI. In our sample where the C allele frequency was only 8%, that variant is unlikely to account for a consistent effect on obesity.

With respect to the *LEP* c.-2548G>A polymorphism, the allele frequency observed in our case series is in accordance with the frequencies found in other studies (Li et al., 1999; Mammès et al., 1998, 2000; Skibola et al., 2004; Snoussi et al., 2006) except for one population from Taiwan, where the *G* allele frequency was somewhat lower than that of the *A* allele (Wang et al., 2006).

The *LEP* c.-2548G>A variant may influence the gene expression of leptin and the leptin secretion by adipose tissue (Hoffstedt et al., 2002), and has been associated with a BMI increase (Li et al., 1999; Mammès et al., 2000). In women with excess weight, this *LEP* variant has also been associated with differences in weight reduction, those with the polymorphism showing a greater difficulty in losing weight (Mammès et al., 1998). Our results showed that the c.-2548G>A polymorphism has no effect on the BMI of obese subjects on spontaneous diet, and are in accordance with data recently published (Skibola et al., 2004; Poitou et al., 2005).

Of the genes studied, only the *LEPR* p.Q223R variant was significantly associated with BMI. Genotype and allele frequencies observed in our study were similar to those found in other populations (Gotoda et al., 1997; Mattevi et al., 2002). In our study, the genotype frequency differed significantly between the lean and obese subjects. This finding may be explained by the significantly greater number of the *AG* genotype in the obese patients, when compared to that of lean individuals. Contrariwise, data from Finland and Korea have shown a higher frequency of the *G* allele in both lean and obese subjects (Salopuro et al., 2005; Park et al., 2006).

The effects of leptin on satiety and energy expenditure are mediated by its hypothalamic receptors (Zhang et al., 1994; Inui, 1999). Exonic DNA sequence variations in the leptin receptor gene may cause a disruption in the leptin-signaling pathway, contributing to common forms of human obesity (Chagnon et al., 1999; Yiannakouris et al., 2001). Our study showed that when the *LEPR* was a variant form, there was a significant association with obesity even with BMI values below 25 kg/m<sup>2</sup>, which was maintained after adjustments for sex and age. These results are in consonance with those carried out in Canadian families (Chagnon et al., 1999) and with those conducted in a Greek population (Yiannakouris et al., 2001). In both studies, there was a higher frequency of the *G* allele. Other investigations have also reported significant associations between the *LEPR* p.Q223R variant and obesity-related phenotypes (Chagnon et al., 2000; Ukkola et al., 2000; Quinton et al., 2001; Mattevi et al., 2002). In two of these studies, one conducted in a group of monozygotic twins from the USA, and another in a population of postmenopausal Caucasian women from the United Kingdom, there was an association of the *LEPR* p.Q223R variants with BMI, fat mass and the cluster of metabolic abnormalities for the *A* allele but not the *G* allele (Ukkola et al., 2000; Quinton et al., 2001).

The association of the variants p.Q223R, p.K109R, and p.K656N with obesity was extensively studied in three meta-analyses (Heo et al., 2001, 2002; Paracchini et al., 2005). The first two reviews (Heo et al., 2001, 2002) selected 20 studies of different populations including African Americans, Pima Indians, and subjects from Canada, Japan, Denmark, Finland, and England. No statistically significant association was found between *LEPR* polymorphism and anthropometric variables such as BMI and waist circumference. The third systematic review (Paracchini et al., 2005) analyzed the raw pooled data from 18 studies of American, European, Asian, and Oceanian populations. They showed no relationship between the *LEPR* variants and BMI and other anthropometric measurements related to overweight and obesity. The authors of these meta-analysis studies pointed out that the lack of association between *LEPR* polymorphisms and obesity could be due to the complex pathogenesis of obesity, which involves a large number of both genetic and environmental factors (Paracchini et al., 2005). Of

note, these studies were carried out in populations with background variation in other genes, which may mask the effects of the *LEPR* gene (Mattevi et al., 2002).

In our study, the haplotype association of the *LEP* c.-2548G>A and *LEPR* p.Q223R variants was related to a 58% increase in obesity risk. It is possible that interactions between polymorphisms of genes intensively involved in the modulation of energy homeostasis may explain the increase in BMI. Interactions of gene-gene polymorphisms involved in leptin regulation have been previously reported. In addition to being associated with BMI, the interaction between the polymorphisms in the *LEP* and *LEPR* genes increases the risk of non-Hodgkin's lymphoma and also influences insulin plasma concentrations and blood pressure levels (Rosmond et al., 2000; Skibola et al., 2004; Snoussi et al., 2006).

Our results support the hypothesis that the p.Q223R *LEPR* variant is associated with a BMI increase. The haplotype association of c.-2548 G>A *LEP* and p.Q223R *LEPR* polymorphisms was related to a significant increase in obesity risk.

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