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ORIGINAL ARTICLE

Metabolic syndrome and ADRB3 gene polymorphism in severely obese patients from South Italy

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Objective: To evaluate the prevalence of β_3-adrenergic receptor (*ADRB3*) Trp64Arg polymorphism and its relationship with the metabolic syndrome in severe obesity.
Design: Cross-sectional outpatients study.
Patients and methods: In 265 (100 men) severely obese non-diabetic subjects and 78 (25 men) healthy volunteers, genomic DNA was isolated from peripheral leukocytes. In obese patients, plasma concentrations of leptin, lipids, glucose and insulin, the homeostasis model assessment index and blood pressure have been measured. The Trp64Arg mutation was identified with the real-time TaqMan method.
Results: Neither genotype distribution nor allele frequency differed between the two groups. The metabolic syndrome prevalence was 59% in obese subjects, and was higher in men than in women (65 vs 55%: $P=0.03$). The body mass index (BMI) was related to age tertiles ($\beta=0.08$; $P<0.001$; multiple linear regression) in Trp64Arg-positive obese subjects.
Conclusion: We confirm the high prevalence of the metabolic syndrome among severely obese subjects. *ADRB3* polymorphism was significantly related to insulin resistance only in obese male subjects. Moreover, increased BMI was related to age in obese subjects with the *ADRB3* polymorphism.
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Introduction

Overeating and physical inactivity, combined with genetic predisposition, are the main causes of obesity in humans. Efforts to identify obesity genes have traditionally focused on white and brown adipose tissues (Arner, 2000), which play a crucial role in regulating energy storage and fuel mobilization in mammals. The *ADRB3* gene is involved in regulating thermogenesis and lipolysis (Arch and Kaumann, 1993). In fact, its stimulation by β-adrenergic agonists activates adenylate cyclase, which, in turn, increases intracellular concentrations of cyclic AMP thereby causing increased lipolysis in white adipose tissue and heat production in brown adipose tissue (Zaagsma and Nahorski, 1990; Lofontan and Berlan, 1993; Katzmarzyk *et al*., 1999).

In humans *ADRB3* is highly expressed in visceral fat (Krief *et al*., 1993) and is responsible for increases in lipolysis and the delivery of free fatty acid into the portal vein (Lönnqvist *et al*., 1995). A mutation in codon 64 of *ADRB3* leads to replacement of tryptophan by arginine (Trp64Arg), which is located in the first intracellular loop of *ADRB3*, and might affect binding to noradrenalin and coupling to G proteins in adipose cells (Walston *et al*., 1995). Therefore the Trp64Arg mutation appears to affect at least some of the receptor's functions *in vivo (*Candelore *et al*., 1996; Pietri-Rouxel *et al*., 1997; Snitker and Odeleye, 1997) and *in vitro (*Hoffstedt *et al*.,

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1999), which would account for decreased lipolysis in white adipose tissue (Katzmarzyk *et al*., 1999).

The Arg64 variant of the *ADRB3* gene has been associated with an earlier onset of type II diabetes mellitus and a decreased acute insulin response to glucose (Walston *et al*., 2000). The pancreas is a major site of *ADRB3* gene expression, and cell transfection with Arg64 was associated with a decrease in glucose-dependent insulin secretion (Perfetti *et al*., 2001). *ADRB3* gene is therefore involved in several metabolic pathways which could be directly or indirectly linked to energy balance and body weight regulation. However, the association between the Trp64Arg *ADRB3* polymorphism and obesity is still controversial, in particular, in Caucasian populations. About half the studies identified an association between the mutation and excess body fat mass (Kadowaki *et al*., 1995; Walston *et al*., 1995; Widen *et al*., 1995; Yoshida *et al*., 1995; Luke *et al*., 1997), whereas the other half did not (Awata and Katayama, 1996; Gagnon *et al*., 1996; Ueda *et al*., 1997; Shiwaku *et al*., 1998). The Trp64Arg mutation was found to predict a greater tendency for abdominal adiposity and high blood pressure with advancing age in a large working population of males from South Italy (Strazzullo *et al*., 2001). It seems to have a weak effect on actual body mass index (BMI); indeed, only a barely statistically significant difference emerged from meta-analyses (Allison *et al*., 1998; Fujisawa *et al*., 1998; Kurokawa *et al*., 2001). These divergent results may be, at least in part, due to population sampling variations, differences in ethnicity, sex, age and degree of excess body fat.

Morbid obesity (BMI $>40\,kg/m^2$), which is increasing in adults and children (Flegal *et al*., 2002), has a strong genetic component (Price *et al*., 1990; Adams *et al*., 1993). Consequently, there patients may represent a good sample in which to evaluate the role of *ADRB3* polymorphisms in obesity predisposition.

Of the two studies that looked for a link between the *ADRB3* Trp64Arg polymorphism and severe obesity, one identified an association (Clement *et al*., 1995) and the other did not (Oksanen *et al*., 1996). Furthermore, the association between the metabolic syndrome (Grundy *et al*., 2005) and the *ADRB3* polymorphism has not been evaluated in severely obese subjects.

We have investigated the association of the Trp64Arg polymorphism with obesity-related phenotypes, in particular, with the metabolic syndrome in a homogenous population of severely obese ($BMI>40\,kg/m^2$) subjects from South Italy. Our aim was to determine the prevalence of the Trp64Arg mutation in severe obesity, and to establish whether this mutation is related with the metabolic syndrome.

Subjects and methods

Subjects

The study population consisted of 265 (100 men, 165 women) severely obese non-diabetic subjects (type III obesity) recruited from the outpatient obesity clinic of the Department of Internal Medicine, 'Federico II' University Hospital in Naples. All subjects had normal liver, kidney and thyroid functions, and none had a history of excessive alcohol intake. No subject was taking antihypertensive drugs or substances known to affect basal metabolic rate, or glucose or lipid metabolism. We evaluated the frequency of the *ADRB3* Trp64Arg polymorphism in the obese subjects and in 78 (25 men 53 women) healthy normal weight volunteers recruited from blood donors living in the same area of South Italy. Obese subjects and controls gave their informed consent to the study, which was approved by the Ethics Committee of our University Hospital.

Anthropometric and metabolic measurements

Weight and height were measured and the BMI calculated as the body weight (kg) divided by the squared height (m^2). Systolic and diastolic blood pressures were measured after the subject had rested for 5 min in a sitting position. After a 10-h overnight fast, 20 ml of blood were collected and immediately centrifuged at room temperature. Plasma glucose was measured by the hexokinase method adapted for an autoanalyzer (Hitachi Modular, Tokyo, Japan). Total cholesterol, triglycerides and high-density lipoprotein (HDL) cholesterol were determined by standard enzymatic methods (Hitachi Modular). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula (Friedewald *et al*., 1972). Serum insulin was measured by the chemiluminescence method (Immulight 2000; Medical System, Genova, Italy). Serum leptin concentrations were measured in duplicate in obese and control individuals with a human leptin enzyme immunoassay (Linco Research, St. Charles, MO, USA). Insulin resistance was estimated according to the homeostasis model assessment (HOMA-IR) method from fasting glucose and insulin concentrations, according to the formula: insulin (mU/ml) $\times$ glucose (mmol/l)/22.5 (Matthews *et al*., 1985). Metabolic syndrome was diagnosed considering the combination of three out of five risk factors according to AHA criteria (Grundy *et al*., 2005).

Genotyping

Genomic DNA was extracted from peripheral leukocytes with a widely used procedure (Nucleon BAAC-2, Amersham, Little Chalfont, Buckinghamshire, UK). The *ADRB3* Trp64Arg polymorphism was identified by the real-time TaqMan method (Applied Biosystems, Foster City, CA, USA) and two fluorescent probes: one specific for the wild-type allele, labeled with the VIC fluorescent reporter dye, and the other complementary to the mutant allele, labeled with the FAM marker. We used the Primer Express program to design the polymerase chain reaction (PCR) primers forward 5′-GCAACCTGCTGGTCATCGT-3′, reverse 5′-GTTGGTCATGGT CTGGAGTCT-3′; MGB TaqMan probes VIC-CATCGCCTGGA CTC-Q-MGB and FAM-ATCGCCCGGACTC-Q-MGB where

MGB is the minor groove binder, a molecule that stabilizes the duplex DNA probe thereby increasing the ability of the hybridization probe to discriminate the single-nucleotide polymorphism). Reaction mixtures were assembled in a 384-well plate using a Biomek 2000 Workstation (Beckman Instruments, Fullerton, CA, USA). Each consisted of: 40 ng of genomic DNA, 36 nM primers, 8 nM probes and 2.5 μl TaqMan Universal Master Mix (Applied Biosystems) in a total reaction of 5 μl. Negative and positive controls (i.e. no DNA, homozygote and heterozygote samples for the Trp64Arg previously typed by sequence analysis on ABI Prism 3100 Genetic Analyzer, Applied Biosystems) were also tested for internal quality control. Real-time PCR was carried out on an ABI Prism 7900 HT instrument with the Sequence Detection System (SDS 2.1) and the SDS Enterprise Database (Applied Biosystems). The amplification protocol consisted of: 50°C for 2 min; 95°C for 10 min; 92°C for 15 s, 60°C for 1 min for 40 cycles; final extension at 60°C for 1 min; final soak at 25°C. The genotypes were determined without prior knowledge of the subjects' status (Afonina *et al*., 1997; Livak, 1999; Kutyavin *et al*., 2000).

Statistics

Descriptive summary statistics for continuous variables are expressed as mean $\pm$ s.d. Before inferential analysis, data on continuous variables were normalized with logarithm transformation. Allele frequencies for each polymorphic site were estimated by the gene-counting method. We used the χ^2 test to compare genotype frequencies. A multiple linear regression analysis, adjusted for sex as potential confounder, was carried out to examine whether the relationship between age and BMI depended on *ADRB3* genotype. We used linear regression analyses (with a control for the effects of age, gender and BMI) to examine the association of the other continuous variables (leptin, lipids, glucose, insulin, HOMA index and blood pressure). The association was analyzed using an additive model in which the differences (coefficient β) are expressed as a function of the number of copies of the more common allele. The interaction between genotype (wild type or the *ADRB3* variant) and gender for biochemical measures of metabolic syndrome was tested with a generalized linear model in which genotype was the fixed variable and gender, age and BMI covariate factor as appropriate. We carried out also a principal component analysis using the correlation matrix of the variables related to insulin resistance dyslipidemia (insulin, triglycerides and HDL cholesterol). According to a generalized linear model adjusted for BMI, ANOVA was carried out with the score calculated from the first principal component accounting for 51% of the variance. The Statistical Package of Social Sciences (SPSS Inc., Chicago, IL, USA) for Windows, version 12.0 was used for statistical analyses.

Results

The mean age of obese subjects was 32. 6 $\pm$ 11.6 years and their mean BMI was 48.2 $\pm$ 7.5 kg/m^2.

Table 1 lists genotype distributions and allele frequencies of the *ADRB3* Trp64Arg polymorphism in obese patients and controls. There were no homozygotes. Neither genotype distribution nor allele frequency differed between obese subjects and controls or between men and women. Genotype frequencies were in accordance with Hardy–Weinberg expectations in the obese ($P = 0.99$) and control ($P = 0.99$) groups.

At multiple linear regression analysis, BMI was significantly related to age ($\beta = 0.08$; $P < 0.001$) only in patients carrying the Trp64Arg mutation. The prevalence of the metabolic syndrome was 59% in the group of obese subjects, and was significantly higher in males than in females (65 vs 55%: $P = 0.03$). Table 2 shows the results of the multiple linear regression analysis of the association between the metabolic variables measured and *ADRB3* genotype in patients. LDL cholesterol was slightly higher in the wild-type group approaching statistical significance ($P = 0.06$); no significant differences were observed between wild-type and carriers.

Figure 1 shows the results of monovariate (panels a–c) and multivariate (d) analyses of the effect of the gender–genotype interaction on insulin resistance and related dyslipidemia. Only in the patients with the Trp64Arg

Table 1 Genotypes and allele frequencies of the Trp64Arg *ADRB3* polymorphism in obese patients

	Obese subjects			*Controls*			P**
	Men (n = *100*)	*Women* (n = *165*)	P*	*Men* (n = *25*)	*Women* (n = *53*)	P*	
ADRB3 Trp64Arg							0.40
Trp/Trp	96 (96%)	156 (94.6%)		24 (96%)	48 (90.6%)		
Trp/Arg	4 (4%)	9 (5.4%)		1(4%)	5(9.4%)		
Arg/Arg	0	0	0.82	0	0	0.70	
Allele frequencies							0.26
Trp64	0.98	0.97		0.98	0.95		
Arg64	0.02	0.03	0.61	0.02	0.05	0.46	

Values were compared with χ^2 analysis; *Men vs Women; **Obese vs Controls.

Table 2 Multiple linear regression analysis of the association between metabolic variables and *ADRB3* genotypes in obese patients

	ADRB3 *genotypes*		*Multiple linear regression analysis*	
	Trp/Trp (n)	*Trp/Arg* (n)	*Beta (95% CI)*	P
Glucose (mmol/l)	5.22±1.147 (229)	4.93±0.50 (13)	0.05(−0.03–0.14)	0.21
HDL cholesterol (mmol/l)	1.16±0.28 (229)	1.13±0.28 (13)	0.03 (−0.01–0.15)	0.69
HDL/total cholesterol (%)	25.1±6.5 (229)	27.3±11.9 (13)	−0.04 (−0.19–0.11)	0.59
LDL cholesterol (mmol/l)	1.29±0.12 (229)	1.18±0.57 (13)	0.16 (−0.01–0.33)	0.06
Triglycerides (mmol/l)	1.46±0.70 (229)	1.48±1.34 (13)	0.09 (−0.17–0.36)	0.49
Insulin (pmol/l)	130.8±65.4 (233)	164.4±231.0 (13)	0.15 (−0.13–0.43)	0.29
HOMA	5.1±2.9 (231)	6.0±8.3 (13)	0.21 (−0.10–0.51)	0.18
Leptin (ng/ml)	110.9±63.0 (149)	112.1±54.8 (7)	0.13(−0.31–0.57)	0.55
SBP (mm Hg)	126±16 (246)	130±13 (12)	−0.03 (−0.09–0.03)	0.39
DBP (mm Hg)	80±10 (246)	81±8 (12)	−0.01 (−0.08–0.06)	0.77

Abbreviations: DBP, diastolic blood pressure; SBP, systolic blood pressure.
The numbers in parentheses are the number of patients; values are not adjusted mean±s.d. (*n*). Differences (β) between genotypes are adjusted for age, sex and BMI in generalized equation models. Differences were analyzed using an additive model and are expressed per copy of the most frequent allele (the allele listed first), in terms of the natural logarithm of the relevant variable.

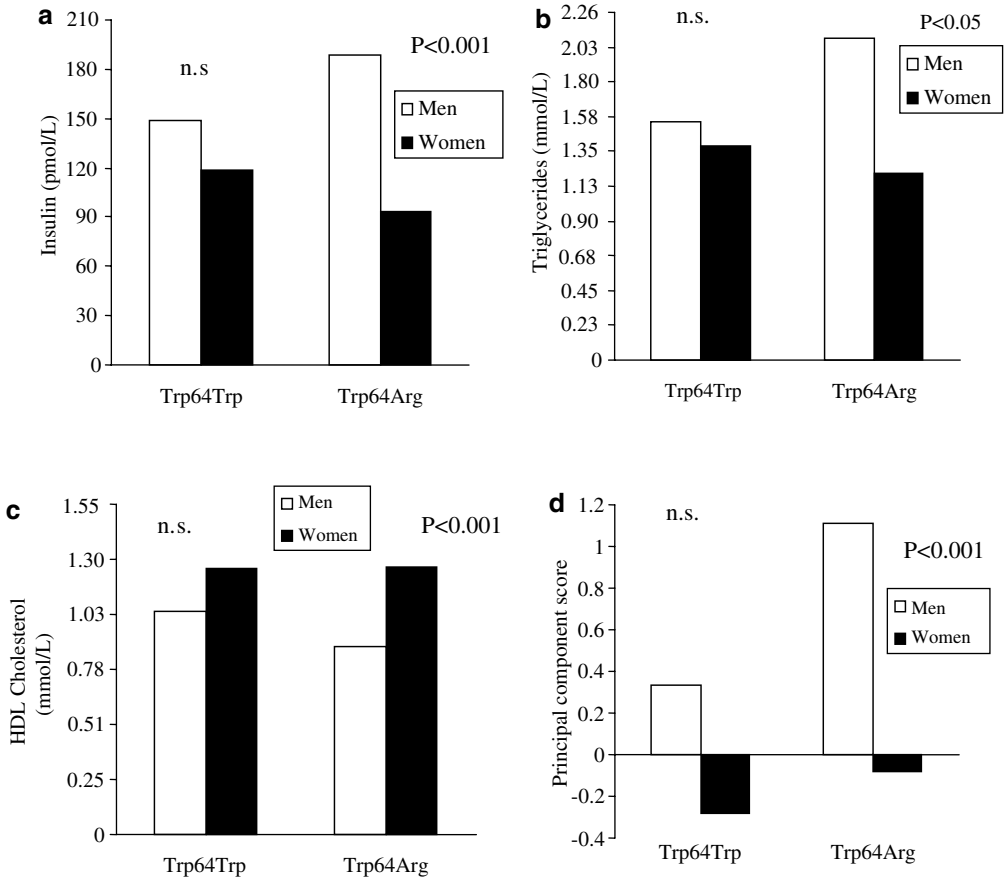

Figure 1 Monovariate (**a–c**) and multivariate (**d**) analysis of the effect of the gender–genotype interaction on insulin resistance-related dyslipidemia. In males with severe obesity ($n=100$: wild type, $n=96$; heterozygote, $n=4$), concentrations of insulin ($P<0.001$) and triglyceride ($P<0.05$) were significantly higher and HDL cholesterol significantly lower ($P<0.001$) than in obese females ($n=165$: wild type, $n=156$; heterozygote, $n=9$) (**a–c**). The differences were more marked in Trp64Arg-positive subjects; and the gender–genotype interaction was statistically significant ($P<0.05$) for insulin. Principal component analysis of the three variables was calculated using a component that accounts for 51% of variance. The principal component score, which reflects insulin resistance, was higher in obese males Trp64Arg carriers ($P<0.001$), with a significant gender–genotype interaction ($P<0.05$) (Figure 1d).

genotype, insulin and triglyceride levels were significantly higher ($P<0.001$ and $P<0.05$, respectively), and HDL cholesterol was significantly lower ($P<0.001$) in men than in women (panels a–c). The interaction between gender and genotype was statistically significant only for insulin ($P=0.037$). The principal component analysis of the three variables (insulin, triglycerides and HDL) was calculated using a component that accounts for 51% of variance. The principal component score, which reflects insulin resistance, was significantly higher in obese males Trp64Arg carriers ($P<0.001$), with a significant gender–genotype interaction ($P<0.026$) (Figure 1d).

Discussion

This study was carried out in a population of severely obese patients, where a strong polygenic component is expected (Price *et al*., 1990; Adams *et al*., 1993) thus representing a target population for genetic studies in human obesity (Clement *et al*., 1995; Oksanen *et al*., 1996).

The prevalence of the *ADRB3* Trp64Arg polymorphism in our obese subjects did not differ from that in the control subjects or from the prevalence already identified in adult male employers of the Olivetti plant in South Italy (Strazzullo *et al*., 2001). In the Québec Family Study cohort (1268 individuals from French-Canadian families) the allelic frequency for the carriers and non-carriers of Trp64Arg were 0.92 and 0.08, respectively (Gagnon *et al*., 1996). In a subsample of obese women (37–60 years old; BMI $>30\,kg/m^2$) of this Canadian population, the allelic frequency of the Arg64 was 0.07 versus 0.04 in controls (Gagnon *et al*., 1996). Furthermore, no significant difference in the frequency of the Trp64Arg polymorphism was observed between obese and lean subjects in German and Finnish studies (Oksanen *et al*., 1996; Evans *et al*., 2000). In particular, the frequency of the Arg64 allele did not differ in Finnish subjects with severe obesity (BMI $\geqslant 40\,kg/m^2$; 9.1%) and in lean healthy control subjects (8.9%) (Oksanen *et al*., 1996).

In our study, there were no Arg64Arg homozygote in either the obese or the control group, and heterozygote were $\sim$5% in both groups in accordance to the literature.

Our data show that Trp64Arg polymorphism is significantly related to weight gain with age thus confirming the results of the Olivetti study carried out in a male working population from the same geographical area (Strazzullo *et al*., 2001). Overall, these observations are in line with the finding that low fat oxidation over time predicts a mild, progressive increase of body fat (Seidell *et al*., 1992; Astrup *et al*., 1997; Marra *et al*., 2004). It is therefore conceivable that impaired *ADRB3* activity may promote body weight gain by reducing lipolysis in adipose tissue and possibly by reducing thermogenesis, that is reduced as a consequence of aging. Indeed an impairment with age of adrenergic-related thermogenesis could be further increased by *ADRB3* polymorphism (Seals and Bell, 2004).

More convincing association between *ADRB3* polymorphism and BMI has been reported in Asian population where the prevalence of *ADRB3* polymorphism is higher than in Caucasian population (Fujisawa *et al*., 1998; Thomas *et al*., 2000; Kurokawa *et al*., 2001).

In our severely obese subjects, the *ADRB3* polymorphism was not significantly associated with the metabolic syndrome, apart from insulin resistance related dyslipidemia in males. Cross-sectional analyses based on data from the Québec Family Study did not reveal any difference in glucose, insulin and resting blood pressure values between carriers versus non-carriers of the Trp64Arg mutation (Gagnon *et al*., 1996). On the other hand, in Japanese subjects it has been reported that the *ADRB3* polymorphism has been associated with insulin resistance but not with dyslipidemia (Kadowaki *et al*., 1995).

The data about the effect of the *ADRB3* mutation on serum triglycerides appear not conclusive. In three studies, the Arg64 allele was unrelated to the plasma lipid profile (Widen *et al*., 1995; Moriarty *et al*., 1997; Ukkola *et al*., 2000). Another study on the mutation carried out in young healthy Danes reported an increase in triglycerides and LDL cholesterol in Arg64 homozygotes (Urhammer *et al*., 1996). In a Spanish study, *ADRB3* gene variants were significantly related to triglyceride and total cholesterol concentrations in men (Corella *et al*., 2001).

In conclusion in our population of severely obese patients the prevalence of the *ADRB3* polymorphism did not differ from that of the control group. On the other hand, the polymorphism was associated in obese patients with a significantly higher age-related BMI. The prevalence of MS was as expected, quite high in our obese subjects and irrespective of the *ADRB3* gene polymorphism. However, insulin resistance was found higher in obese males carrying the *ADRB3* mutation. Further data from prospective studies are required to clarify the relationship between aging and increased BMI as related to *ADRB3* polymorphism as well as other thermogenesis-related genes.

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