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ORIGINAL ARTICLE

Association of a beta-2 adrenoceptor (*ADRB2*) gene variant with a blunted *in vivo* lipolysis and fat oxidation

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Background and aims: Obesity is associated with a blunted β-adrenoceptor-mediated lipolysis and fat oxidation. We investigated whether polymorphisms in codon 16, 27 and 164 of the β_2-adrenoceptor gene (ADRB2) and exon 10 of the G protein β_3-subunit gene (GNB3) are associated with alterations in *in vivo* lipolysis and fat oxidation.
Design and methods: Sixty-five male and 43 female overweight and obese subjects (body mass index (BMI) range: 26.1–48.4 kg/m^2) were included. Energy expenditure (EE), respiratory quotient (RQ), circulating free fatty acid (FFA) and glycerol levels were determined after stepwise infusion of increasing doses of the non-selective β-agonist isoprenaline (ISO).
Results: In women, the Arg16 allele of the *ADRB2 gene* was associated with a blunted increase in circulating FFA, glycerol and a decreased fat oxidation during ISO stimulation. In men, the Arg16 allele was significantly associated with a blunted increase in FFA but not in glycerol or fat oxidation.
Conclusion: These results suggest that genetic variation in the *ADRB2 gene* is associated with disturbances in *in vivo* β-adrenoceptor-mediated lipolysis and fat oxidation during β-adrenergic stimulation in overweight and obese subjects; these effects are influenced by gene–gender interactions.
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Keywords: lipolysis; fat oxidation; single-nucleotide polymorphism; *ADRB2* gene; *GNB3* gene

Introduction

Obesity is characterized by increased circulating free fatty acid (FFA) concentrations and increased triglyceride (TG) storage within adipose tissue. *In vivo* studies have shown that the development or maintenance of increased adipose tissue stores might be promoted by a blunted lipolytic response and fat oxidation after β-adrenergic stimulation or exercise in obese or obese type 2 diabetic subjects.[1–4] This blunted β-adrenoceptor-mediated lipolysis and fat oxidation persisted after weight reduction, indicating that this disturbance may be an early, even primary factor, in the development or maintenance of increased adipose stores.[1] There are indications that the blunted β-adrenergically mediated lipolysis in obesity may be related to an impaired function or a reduced number of adipocyte beta-2 (β_2) adrenoceptors.[5,6]

β_2-Adrenoceptors are stimulatory G-protein-coupled (G_s) receptors. The β_2-adrenoceptor (*ADRB2*) gene is encoded by an intronless gene on chromosome 5q31–q32.[7,8] Several polymorphisms of the human *ADRB2* gene have been described.[9,10] Among these, three common single-nucleotide polymorphisms (SNPs) result in the substitution of an amino acid. One is located at codon 16, substituting arginine for glycine (Arg16Gly). The other one is located at codon 27, substituting glutamic acid for glutamine (Gln27Glu). Both variants are located in the extracellular amino-terminal region of the receptor and alter cellular trafficking and desensitization of the receptor.[11] Previous studies have reported associations between codons 16 and 27 polymorphisms and obesity, insulin resistance and hypertension.[12–22] Finally, the substitution of isoleucine for threonine at codon 164 (Thr164Ile), in the receptor transmembrane-spanning domains, alters agonist binding and decreases coupling of the G_s protein to the receptor.[23,24] There is evidence from *in vitro* studies that some of these receptor variants might be important for catecholamine-induced adipocyte lipolysis in humans.[16,25]

Furthermore, polymorphisms in G proteins involved in catecholamine signaling may alter corresponding receptor and hormone function. Recently, a common polymorphism substituting a cytosine for a thymine at position 825 (C825T) in exon 10 of the G-Protein β_3-subunit (*GNB3*) gene

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(chromosome 12p13), coding an isoform of the G protein β subunit ($G\beta_3$), has been identified.[26] G_s deficiency is also observed in obesity and the C825T polymorphism is associated with obesity and hypertension in both white and non-white populations.[27–34] *In vitro* studies indicated that the 825T variant of $G\beta_3$ in its homozygous form was associated with a decreased amount of $G\beta_3$ in fat cells, thereby inhibiting signaling through β_1-, β_2- and α_2-adrenoceptors, resulting in decreased catecholamine action and blunted lipolysis in isolated subcutaneous adipocytes of male and female obese subjects.[35]

Thus, there are indications that polymorphisms in the *ADRB2* gene and the *GNB3* gene may be related to an impaired *in vitro* lipolytic response. So far, however, few *in vivo* lipolysis studies on these polymorphisms have been performed. For this reason, the present study investigated the effect of genetic variation in the *ADRB2* and the *GNB3* gene on *in vivo* lipolysis and fat oxidation in overweight and obese subjects.

Methods

Subjects

The study group consisted of 108 overweight and obese (BMI range: 26.1–48.4 kg/m^2) subjects (43 F/65 M). Twenty-four overweight subjects (BMI between 25 and 29.9 kg/m^2; 11 F/13 M) and 84 obese subjects (BMI > 29.9 kg/m^2; 32 F/52M) were included. The basic selection criteria were age 20–50 years and BMI > 25 kg/m^2. Exclusion criteria were weight change > 3 kg within 3 months before the study started, drug-treated hypertension, diabetes or hyperlipidemia, thyroid disease, surgically treated obesity, pregnancy, alcohol or drug abuse and participation in other simultaneous ongoing trials. All subjects were recruited by means of an advertisement in a local newspaper. All subjects were in good health as assessed by medical history and physical examination and were not taking any medication. A normal resting electrocardiogram (ECG) and blood pressure were a prerequisite for participation. The study protocol was reviewed and approved by the Medical Ethical Review Committee of Maastricht University. The subjects were informed in detail about the investigation and their consent was obtained before participating in the study.

Anthropometric measurements

Body weight was determined on an electronic scale, accurate to 0.1 kg. Waist and hip circumference measurements to the nearest 1 cm were made with the subjects standing upright. BMI was calculated as body weight in kilograms divided by squared height in meters. Body density was obtained by underwater weighing with residual pulmonary volume measurement by the helium dilution method (Volugraph 2000, Mijnhardt) and was converted to percent body fat using the equation of Siri.[36] Fat mass (FM) and fat-free mass (FFM) were calculated from the percent body fat and body weight.

Study design

The subjects arrived at the laboratory at 0800 after an overnight fast (of at least 12 h) by car or public transport. They were studied while resting supine on a comfortable bed in a room kept at 23–25°C. At the beginning of the experiment, a catheter was inserted into a forearm vein for blood sampling. A second catheter was inserted into the contralateral arm for infusion of the non-selective β-agonist isoprenaline (ISO). Thirty minutes after insertion of the catheters, the measurement protocol started. Energy expenditure and substrate oxidation were measured during the entire period with an open-circuit ventilated hood system. After 30 min, blood was sampled for baseline measurements and genetic analysis. Following the 30-min baseline period, ISO infusion started at increasing concentrations of 6, 12 and 24 $ng \cdot kg\,FFM^{-1} \cdot min^{-1}$, each dose for 30 min. At the end of each infusion period, venous blood samples were taken, centrifuged and stored at −80°C until further analysis. During ISO infusion, heart rate was kept under close observation by means of an ECG. When the heart rate increased by more than 30 beats/min above baseline or in case of an irregular heart rhythm, ISO infusion was stopped.

Genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes by digestion with protein K followed by phenol/chloroform extraction. Amplification of the relevant segments of the *ADRB2* and *GNB3* genes was performed by polymerase chain reaction (PCR) as previously described.[16,35] PCR products were digested at 37°C for 1 h using *Bsr*DI, *Ita*I, *Mnl*I or *Bse*DI. The digested fragments were visualized using ethidium bromide staining and UV-transmitted light. Finally, we evaluated the accuracy of the restriction fragment length polymorphism (RFLP) method by direct sequencing of random samples and got 100% agreement. In addition, two persons independently evaluated samples and identical results were obtained.

Biochemical analysis

Whole blood was collected in tubes containing ethylene diamine tetraacetic acid (EDTA) and centrifuged for 10 min at 3000 rpm (4°C) and the plasma removed for the enzymatic calorimetric quantitation of FFA (NEFA C kit, Wako, Neuss, Germany) and glycerol (Boehringer, Mannheim, Germany) on a COBAS FARA centrifugal spectrometer (Roche Diagnostica). Standard samples with known concentrations were included in each run for quality control.

Statistical analysis
All statistical calculations were performed with SPSS for Macintosh (version 11.0; SPSS Inc., Chicago, IL, USA). The initial statistical analysis, performed for the whole group ($n=108$), indicated a significant gender effect for the Arg16 allele on lipolytic parameters (iAUC ΔFFA; $P=0.01$, allel–gender interaction) and fat oxidation (iAUC ΔRQ; $P=0.042$). For this reason, the presented analysis is stratified by gender. The effect of genotypes on lipolytic responses and fat oxidation was investigated using analysis of variance (ANOVA) (adjusted for age and BMI). *Post hoc* testing was performed by Student's unpaired *t*-test with Bonferroni correction. Linkage disequilibrium was estimated according to Devlin *et al*.[37] Diplotype analysis were performed as described before.[38,39] The goodness of fit between observed and expected genotype frequency (Hardy–Weinberg equilibrium, HWE) was statistically tested using the χ^2-test.[40] Allele and genotype frequency distributions for the whole group ($n=108$) are presented in Table 2. Energy expenditure (EE) was adjusted for FFM by means of covariance analysis (ANCOVA). The ISO-induced effect on fat oxidation (RQ), thermogenesis (EE) and lipolysis (FFA, glycerol) were expressed as incremental area under the curve (iAUC) above baseline, calculated according to the trapezium rule. All data are represented as mean±standard error of the mean (s.e.m.). $P<0.05$ was considered as statistically significant.

Power calculation
A power analysis was performed to estimate the sample size required enabling the accurate and reliable statistical judgments for the two-way parametric statistics. There are little or no published studies regarding the effect of *ADRB2* and *GNB3* gene variants and whole body lipolysis and fat oxidation, although the results of *in vitro* studies in human adipocytes support a major effect. We therefore estimated our sample size using published data of the effect of *β*-adrenergic stimulation on whole body lipolysis and fat oxidation.[4,41,42] Power calculation indicated that to detect a difference in circulating FFA of 100 μmol/l (with an s.d. of 50 μmol/l) or circulating glycerol of 50 μmol/l (with an s.d. of 25 μmol/l) and a power of 0.80 ($\alpha=0.05$ and $\beta=0.20$), the number of subjects in each group (two-tailed) should be 16.

Results

As mentioned under statistical methods, initial analysis performed for the whole group ($n=108$) indicated a significant gender effect for the Arg16 allele. For this reason, the presented analysis is stratified by gender.

Subjects
Anthropometric and metabolic characteristics of the study subjects are shown in Table 1. Women had significantly higher percentage body fat, a lower waist–hip ratio and were significantly younger compared with male participants. Additionally, women had a significantly higher fasting FFA level. No significant differences were observed for BMI, resting EE (adjusted for FFM) and fasting glycerol levels between genders.

*Effect of codon 16 and 27 of the β_2-adrenoceptor gene (*ADRB2*) on fat oxidation and lipolysis after* β*-adrenergic stimulation*
Allele and genotype frequency distributions for the *ADRB2* gene are shown in Table 2. For women, ANOVA analysis

Table 1 Subject characteristics

	Men (n = *65*)	*Women* (n = *43*)	P-*value*
Age (year)	43.3±1.0	38.8±1.3	*
BMI (kg/m^2)	32.5±0.5	32.3±0.6	NS
% body fat	31.7±0.7	42.4±0.7	**
WHR	1.04±0.01	0.86±0.02	**
EE resting (kJ/min) #	5.56±0.10	5.72±0.13	NS
RQ resting	0.81±0.01	0.82±0.01	NS
Fasting FFA (μmol/l)	477±27	607±30	**
Fasting glycerol (μmol/l)	73±3	77±7	NS

All values are means±s.e.m. Abbreviations: BMI, body mass index; EE, energy expenditure; FFA, free fatty acids; RQ, respiratory quotient; WHR, waist-to-hip ratio;,. # EE, adjusted for FFM. *$P=0.01$, **$P<0.001$ men vs women using Student's unpaired *t*-test.

Table 2 Allele, genotype and diplotype frequency distributions of the *ADRB2* gene and *GNB3* gene polymorphisms

	n = *108*
Allele frequency	
Arg16	0.394
Gln27	0.569
Thr164	0.972
C	0.741
Genotype frequency	
Gly16Gly (wt)	0.371
Arg16Gly	0.472
Arg16Arg	0.157
Gln27Gln (wt)	0.352
Gln27Glu	0.435
Glu27Glu	0.213
Thr164Thr (wt)	0.944
Thr164Ile	0.056
Ile164Ile	0
CC	0.537
CT	0.407
TT	0.055
Diplotype frequency	
Gly16Gly/Glu27Glu	0.185
Gly16Gly/Gln27Gln	0.018
Arg16Arg/Gln27Gln	0.148

Alleles, genotypes and diplotypes are presented as decimals. All SNPs were in HWE. Abbreviations: Arg, arginine; Gln, glutamic acid; Glu, glutamine; Gly, glycine; Ile, isoleucine; Thr, threonine; wt, wild type.

indicated a significant genotype effect for codon 16 and a blunted increase in FFA ($P=0.046$, see Figure 1), glycerol ($P=0.037$, see Figure 1) and fat oxidation ($P=0.042$, see Figure 2), even after correction for age and BMI. *Post hoc* analysis indicated that in women the Arg16Gly genotype was significantly associated with a blunted increase in circulating FFA (iAUC ΔFFA after ISO: 379 ± 35 vs 493 ± 40 μmol/l, $P=0.041$, see Figure 1) and glycerol (iAUC ΔGlycerol after ISO: 86 ± 11 vs 128 ± 14 μmol/l, $P=0.026$, see Figure 1) during stimulation compared with female Gly16 homozygotes. In addition to a blunted lipolytic response, female Arg16Gly heterozygotes showed a blunted increase in fat oxidation compared with Gly16 homozygotes (iAUC ΔRQ after ISO: 0.004 ± 0.007 vs -0.017 ± 0.008, $P=0.043$, see Figure 2) and a comparable thermogenic response (iAUC ΔEE after ISO: 0.51 ± 0.07 vs 0.70 ± 0.09 kJ/min, NS).

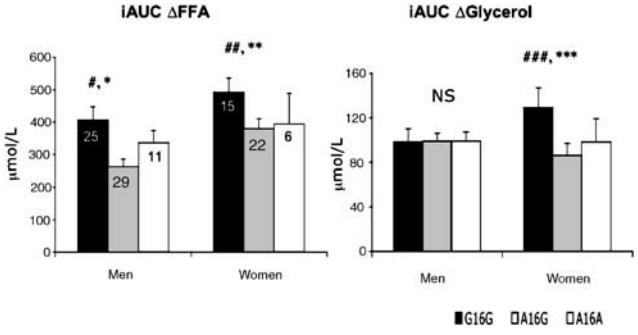

Figure 1 Lipolytic response for codon 16 polymorphisms of the *ADRB2* gene. All values are means ± s.e.m. iAUC ΔFFA, iAUC Δglycerol: incremental area under the curve for circulating free fatty acid (FFA) or glycerol concentration during β-adrenergic stimulation. G16G: Gly16Gly (black bar), A16G: Arg16Gly (gray bar), A16A: Arg16Arg (white bar). ANOVA (adjusted for age and BMI): $^{\#}P=0.022$, $^{\#\#}P=0.046$, $^{\#\#\#}P=0.037$. *Post hoc* Student's unpaired *t*-test: $^{*}P=0.005$, $^{**}P=0.041$, $^{***}P=0.026$ A16G vs G16G. Number of subjects in each group is indicated in the bars.

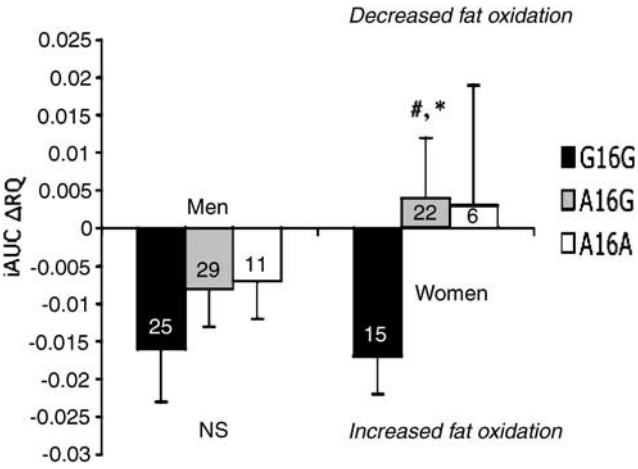

Figure 2 Fat oxidation for codon 16 polymorphisms of the *ADRB2* gene. All values are means ± s.e.m. iAUC ΔRQ: Incremental area under the curve for delta respiratory quotient (RQ) during β-adrenergic stimulation. G16G: Gly16Gly (black bar), A16G: Arg16Gly (gray bar), A16A: Arg16Arg (white bar). ANOVA (adjusted for age and BMI): $^{\#}P=0.042$. *Post hoc* Student's unpaired *t*-test: $^{*}P=0.043$ G16G vs A16G. Number of subjects in each group is indicated in the bars.

Because lipolytic response and fat oxidation appeared to be reduced in both Arg16Gly and Arg16Arg carriers, Arg16 heterozygotes and homozygotes were combined into one group (Arg16Gly + Arg16Arg). Female Arg carriers (Arg16Gly + Arg16Arg) appeared to have a blunted increase in circulating FFA (382 ± 31 vs 493 ± 43 μmol/l, $P=0.042$) and glycerol (89 ± 10 vs 129 ± 18 μmol/l, $P=0.038$) after β-adrenergic stimulation compared with Gly16Gly homozygotes. This altered lipolytic response in female Arg carriers (Arg16Gly + Arg16Arg) was accompanied by a decreased fat oxidation after stimulation (iAUC ΔRQ after ISO 0.003 ± 0.007 vs -0.016 ± 0.005, $P=0.024$). No differences were found in ISO-induced thermogenesis (iAUC ΔEE after ISO: 0.57 ± 0.07 vs 0.70 ± 0.09 kJ/min, NS), body weight, BMI and other anthropometric variables.

Data in male overweight subjects were less consistent: ANOVA analysis (adjusted for age and BMI) indicated only a significant genotype effect of codon 16 and a blunted increase in FFA ($P=0.022$, see Figure 1). *Post hoc* analysis revealed that male carriers of the Arg16Gly genotype had a significantly blunted increase in circulating FFA during β-adrenergic stimulation compared with male Gly16 homozygotes, (266 ± 30 vs 401 ± 34 μmol/l, $P=0.005$; see Figure 1). However, this blunted FFA response was not accompanied by a blunted increase in circulating glycerol (see Figure 1) nor a decreased fat oxidation (iAUC ΔRQ, see Figure 2). Furthermore, taking Arg16 heterozygotes and homozygotes together into one group, (Arg16Gly + Arg16Arg) carriers showed a blunted increase in circulating FFA (281 ± 21 vs 406 ± 41 μmol/l, $P=0.004$) compared with Gly16Gly carriers, whereas ISO-induced changes in glycerol, thermogenesis and RQ were comparable between groups. Again, no differences were found in body weight, BMI and other anthropometric variables.

For neither female nor male subjects associations were found between genetic variation in codon 27 and 164 of the β_2-adrenoceptor gene (*ADRB2*) and alterations in fat oxidation or lipolytic response during β-adrenergic stimulation.

Diplotype analysis

Three homozygous and functional diplotypes were investigated in both male and female subjects: Gly16Gly/Glu27Glu, Arg16Arg/Gln27Gln and Gly16Gly/Gln27Gln. The diplotype frequency distribution is depicted in Table 2. From the 55 men carrying the Gly16 allele, 47 also carried the Glu27 allele ($\chi^2=32.653$, $P<0.0001$), indicating linkage disequilibrium ($|D'|=0.854$, $r^2=0.494$).[37] In 21.5% ($n=14$) of the men and 14% ($n=6$) of the women, the Gly16Gly/Glu27Glu diplotype was apparent. This diplotype was not associated with a decreased lipolytic response (iAUC ΔFFA and iAUC Δglycerol) or fat oxidation (iAUC ΔRQ) in male or female

subjects. Nevertheless, female carriers ($n=6$) of the Gly16Gly/Glu27Glu diplotype had lower fasting FFA levels (431 ± 49 vs $636\pm32\,\mu$mol/l, $P<0.05$) compared with female non-carriers ($n=37$). No differences were found in body weight, BMI and other anthropometric variables. The other two diplotypes (Arg16Arg/Gln27Gln and Gly16Gly/Gln27Gln) were also not associated with an altered fat oxidation, thermogenic or lipolytic response in this population. Owing to the relatively low sample size, we were not able to identify β_2-adrenoceptor haplotypes for codons 16, 27 and 164 with a frequency $>5\%$.

Effect of the C825T polymorphism in exon 10 of the G protein beta-3 subunit (GNB3) gene on fat oxidation and lipolysis
Allele and genotype frequency distributions for the *GNB3* gene are shown in Table 2. Male TT carriers ($n=4$) showed a tendency toward a blunted increase in circulating FFA (iAUC ΔFFA after ISO: 173 ± 46 vs $435\pm32\,\mu$mol/l) and glycerol (iAUC ΔGlycerol after ISO: 47 ± 14 vs $107\pm8\,\mu$mol/l) after β-adrenergic stimulation compared with CT carriers ($n=29$). Similar results were obtained for male CC carriers ($n=32$). Unfortunately, the number of subjects in the TT group ($n=4$) were too small to perform statistical analysis. In female overweight subjects, no associations were found for the C825T polymorphism and an altered lipolytic response, thermogenesis or fat oxidation.

Discussion

To the best of our knowledge, this is the first study to investigate the association between genetic variability in the *ADRB2* gene and *GNB3* gene and *in vivo* lipolysis and fat oxidation in overweight and obese men and women.

The major findings of our study are as follows: firstly, genetic variability in codon 16 of the *ADRB2* gene was associated with a blunted increase in circulating FFA and glycerol during β-adrenergic stimulation with the non-selective ISO in female subjects. In male subjects, codon 16 was associated with a blunted ISO-induced increase in FFA, whereas no difference in glycerol was apparent. In female subjects, this blunted lipolytic response was also accompanied with a reduced fat oxidation. Finally, the TT genotype of the *GNB3* gene was associated with a blunted increase in FFA and glycerol in male subjects. This blunted lipolytic response was not accompanied by a reduced fat oxidation.

Large *et al*.[16] showed that the Arg16Gly genotype was associated with an *in vitro* fivefold increase in agonist sensitivity of the β_2-adrenoceptor in abdominal subcutaneous adipocytes of overweight female subjects, without any significant effect on glycerol release. In our study, the Arg16 allele was associated with blunted ISO-induced responses in FFA, glycerol and fat oxidation (iAUC ΔRQ) in women and a blunted increase in FFA in men. The reason for this apparent discrepancy with our findings may be related to the differences in our *in vivo* vs the *in vitro* approach to study lipolysis. In the *in vitro* situation, *in vivo* factors like the neuroendocrine environment and local adipocyte blood flow are not taken into account. In addition, the majority of *in vitro* studies are performed on adipocytes derived from the subcutaneous region in both genders. It should be mentioned that there are major differences in catecholamine-induced lipolysis between depots (subcutaneous vs visceral and gluteofemoral) and also gender differences in body fat distribution.[43–45] Our data indicate that variability in codon 16 of the *ADRB2* gene may contribute to a reduced *in vivo* β-adrenoceptor-mediated lipolysis and fat oxidation,[1–4] indicating that these blunted responses may be important primary factors in obesity.

Besides looking at individual codons, we also studied the effect of diplotypes. We chose to study two common ($>10\%$ in the population) and one less common ($<5\%$) homozygous combination (Gly16Gly/Glu27Glu, Arg16Arg/Gln27Gln and Gly16Gly/Gln27Gln), as they have been reported to have a significant effect on lipolysis.[38] In addition, these SNPs belong to the same pathway and transfection experiments showed that they are functional.[39] Finally, in our population, as has been reported before, there is strong linkage disequilibrium between codons 16 and 27.[46] Nevertheless, no effect of diplotypes on lipolytic, thermogenic response or fat oxidation was found in our study. Only the Gly16Gly/Glu27Glu diplotype was associated with a lower fasting FFA concentration in female overweight subjects, which may possibly reflect a reduced rate of lipolysis in subcutaneous adipose tissue.[47] Finally, literature suggests that the Thr164Ile β_2-adrenoceptor polymorphism is closely associated with Gly at position 16 and Gln at position 27.[39,48] Nevertheless, due to the relatively low sample size, we were not able to identify β_2-adrenoceptor haplotypes for codons 16, 27 and 164 with a frequency $>5\%$.[38]

The observed genotype frequency for the polymorphism in the *GNB3* gene was similar to that previously reported in other Caucasian populations.[26,31] Rydén *et al*.[35] showed that the T variant of this polymorphism was associated with a blunted *in vitro* responsiveness for the non-selective ISO in abdominal subcutaneous adipocytes of male and female overweight subjects. In contrast with Ryden *et al*.[35] we found a tendency toward a reduced lipolysis aney in male overweight subjects. It should be mentioned that our sample has no adequate power to provide a conclusive result about a genotype effect for the C825T polymorphism in the *GNB3* gene. Thus, further studies are necessary to confirm our *in vivo* findings in a larger population.

In summary, variation in codon 16 of the *ADRB2* gene is associated with an impaired lipolytic response in male and female overweight and obese subjects and by a blunted fat oxidation in overweight and obese women. In conclusion, the present results suggest that genetic variability in the *ADRB2* gene influences lipolysis regulation *in vivo* in overweight and obese subjects and that this is subject to gene–

gender interactions. This indicates that genetic variability in the *ADRB2* gene may be an important factor in the development or progression of obesity and obesity-related disorders.

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