Role of Ghrelin Polymorphisms in Obesity Based on Three Different Studies

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Abstract

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Objective: Associations between preproghrelin DNA variants and obesity-related phenotypes were studied in 3004 subjects from the Québec Family Study (QFS), the HERITAGE Family Study (HERITAGE), and the Swedish Obese Subjects (SOS) Study.

Research Methods and Procedures: Body mass index (BMI), fat mass (FM) from underwater weighing, and abdominal fat from computerized tomography were measured. The ghrelin polymorphisms were identified by polymerase chain reaction.

Results: Arg51Gln QFS subjects ($n = 6$) had lower ghrelin concentrations ($p = 0.007$) than Arg51Arg subjects ($n = 14$). White preproghrelin Met72Met subjects in HERITAGE had the lowest BMI ($p = 0.020$), and those in the QFS cohort had the lowest FM ($p < 0.001$). Met72 carrier status (Met72+) was associated with lower FM ($p = 0.026$) and higher insulin-like growth factor-1 levels ($p = 0.019$) among blacks. Met72Met QFS subjects had less visceral fat ($p = 0.002$) and a lower fasting respiratory quotient ($p = 0.037$). HERITAGE Met72+ white subjects also showed lower exercise respiratory quotient ($p = 0.030$) and higher maximal oxygen uptake ($p = 0.023$). Furthermore, the prevalence of Met72+ was higher (19.2%; $p < 0.05$) in SOS subjects whose BMI was ≤ 25 kg/m^2 than in those with BMI >25 kg/m^2 (14.8%). SOS Met72+ obese women had a lower (11.4%; $p = 0.032$) prevalence of hypertension than noncarriers (23.9%).

Discussion: Arg51Gln mutation was associated with lower plasma ghrelin levels but not with obesity. The preproghrelin Met72 carrier status seems to be protective against fat accumulation and associated metabolic comorbidities.

Key words: growth hormone, visceral fat, adiposity, respiratory quotient, hypertension

Introduction

Ghrelin is an endogenous ligand for the growth-hormone secretagogue receptor (1) that stimulates growth hormone release (2–4). Hence, ghrelin action could lead to decreased adiposity through the known lipolytic activity of growth hormone (5). However, recent studies in rodents suggest that peripherally or centrally administered ghrelin, independent of growth hormone, decreases fat oxidation and increases food intake (6) and adiposity (7). The observation that plasma ghrelin levels are lower in obese human subjects (8) is even more intriguing. Ghrelin may participate in meal initiation (9) and signal to the hypothalamus when an increase in metabolic efficiency is needed (6).

The characterization of the gene encoding ghrelin and its overall genomic structure (10) has made possible genomic screening of the ghrelin gene. Mutations in the ghrelin gene could potentially cause a defective or inactive ghrelin protein and alter growth hormone secretion and energy balance.

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We have earlier identified three sequence variants in the preproghrelin/ghrelin gene, two of which were shown to be associated with obesity in a sample of the Swedish Obese Subjects Study (SOS) cohorts (11). We report here the associations of two preproghrelin exonic polymorphisms on obesity-related phenotypes in the Québec Family Study (QFS) and the HERITAGE Family Study (HERITAGE). In addition, a larger sample size from the SOS cohort was used to compare the allele frequencies between obese and nonobese subjects and to verify whether these polymorphisms were associated with morbid obesity.

Research Methods and Procedures

A total of 3004 subjects from the three cohorts was available for this study. The QFS cohort (12) includes individuals from French-Canadian families living in and around Québec City. A subsample of 784 subjects participating in Phase 2 of QFS (from 1989 to 1997) was included in the present study.

The HERITAGE study is a multicenter clinical trial conducted at five institutions. The specific aims, design, inclusion, and exclusion criteria and methodology of the study have been described in detail elsewhere (13). The sample for the present study consists of 778 subjects (502 whites and 276 blacks). To be enrolled in the study, the individuals were required to be healthy (i.e., free of diabetes, cardiovascular diseases, or other chronic diseases) and sedentary at baseline (defined as no regular physical activity over the previous 6 months).

The SOS cohort has been previously described (14,15). Briefly, SOS is an intervention trial designed to determine whether mortality and morbidity rates among obese individuals who lose weight by surgical means differ from those associated with conventional treatment. The SOS study consists of three cohorts: a registry of morbidly obese subjects, a surgical intervention group selected from the registry, and a normal reference population. A total of 1442 subjects (including 741 subjects from the obese registry and 701 subjects from the normal reference population) were included in the present study. Before undergoing a health examination, all subjects completed a series of questionnaires on current and past health status (15). The diagnosis of hypertension, diabetes, or stroke was based on self-reported data collected in questionnaires.

Body Composition Phenotypes

Body mass index (BMI) was calculated as body weight (kilograms) divided by squared height (square meters). In the QFS, body density obtained by underwater weighing (16) was converted to percentage of body fat using the equation of Siri (17), with residual pulmonary volume measured by helium dilution (18). Fat mass (FM) and fat-free mass (FFM) were obtained from percentage of body fat and body weight. Similar procedures were followed to measure body density, from which FM and FFM were estimated (19). Abdominal fat areas in QFS and HERITAGE cohorts were obtained using computerized tomography as described elsewhere (20). In the QFS cohort, respiratory quotient (RQ) was measured using a ventilated hood and an open-circuit indirect calorimeter (21). Measurements were done early in the morning, after an overnight fast, while participants sat quietly in a semireclined position for 30 minutes.

Plasma Lipids and Lipoproteins (QFS and HERITAGE)

After a 12-hour overnight fast, venous blood samples were taken in EDTA tubes for plasma lipid and lipoprotein determinations. Cholesterol and triglyceride concentrations were determined in plasma and lipoprotein fractions using an RA-1000 automated analyzer (Technicon Instruments Corp., Tarrytown, NY) as reported earlier (22). Plasma very-low-density lipoproteins ($d < 1.006$ g/mL) were isolated by ultracentrifugation (23), and the high-density lipoprotein fraction was obtained after precipitation of low-density lipoprotein in the infranatant ($d > 1.006$ g/mL) with heparin and $MnCl_2$, as previously described (24).

Serum insulin-like growth factor-1 (IGF-1) measurements were performed in HERITAGE subjects using the IGF-1 EIA procedure supplied by ALPCO (Windham, NH). Samples were treated with a releasing agent to inactivate binding proteins and pipetted into microtiter plate wells coated with purified sheep polyclonal anti-IGF-1 antibody. They were then incubated with horseradish peroxidase-labeled monoclonal anti-IGF-1. After washing, tetramethyl benzidine substrate was added. The reaction was stopped and the absorbance read at 450 nm. A cubic spline curve was used to determine concentration.

Ghrelin Measurements in QFS

Plasma samples were collected under fasting conditions. Human plasma ghrelin was measured with a commercial radioimmunoassay (Phoenix Pharmaceuticals, Inc., Belmont, CA) that uses ^{125}I-labeled bioactive ghrelin as a tracer molecule and a polyclonal antibody raised in rabbits against human ghrelin. This antibody recognizes total ghrelin (including octanoylated as well as nonoctanoylated human ghrelin). Negligible crossreactivity was found with motilin-related peptide (<1%). No cross-reactivity has been reported with motilin, human secretin, vasoactive intestinal peptide, prolactin releasing peptide-31, galanin, growth hormone releasing hormone, neuropeptide Y, or other relevant molecules.

Cardiorespiratory Fitness in HERITAGE

Two maximal exercise tests were performed on separate days on a cycle ergometer (800S; Ergo-Metrics, Yorba Linda, CA) (25,26). V_{O_2} was determined every 20 seconds and was reported as a rolling average of the three most recent 20-second values. All respiratory variables were

measured with a SensorMedics 2900 metabolic measurement cart (Yorba Linda, CA). $V\text{O}_{2\text{max}}$ was defined as the mean of the highest $V\text{O}_2$ values determined in each of the two maximal tests, or the higher of the two values if they differed by >5%. The intraclass correlation coefficient for repeated measurements of $V\text{O}_{2\text{max}}$ reached 0.97 (26). Exercise RQ ($V\text{O}_2/V\text{CO}_2$ or respiratory exchange ratio) was measured both at 50 W and at 60% of subject's $V\text{O}_{2\text{max}}$ and the intraclass correlation coefficient for repeated measures ranged from 0.58 to 0.72 (27).

Polymerase Chain Reaction Analysis

Genomic DNA was extracted from white blood cells or lymphoblastoid cell lines. The replacement of a guanine (G) with an adenine (A) at base 346 in exon 2 of the preproghrelin gene abolishes the *Sac*I restriction site. The preproghrelin Leu72Met polymorphism is caused by a cysteine (C)-to-A transition at base 408 in exon 2 of the preproghrelin gene that leads to the abolishment of the *Bsr*I restriction site. Two primers were designed to generate a 618 base-pairs product covering exons 1 and 2 encompassing the entire mature ghrelin product. The product was amplified from leukocyte DNA by the polymerase chain reaction (PCR) technique. The primer sequences were as follows: forward primer, 5′-GCTGGGCTCCTACCTGAGC-3′; reverse primer, 5′-GGACCCTGTTCACTGCCAC-3′. The PCR amplification was carried out in a volume of 25 μL containing 150 ng DNA, 0.12 μM of each primer, 0.2 mM of each of the dNTPs (Amersham Pharmacia Biotech Inc., Piscataway, NJ), and 1.0 unit Taq polymerase (Qiagen, Valencia, CA). The PCR was performed at 95 °C for 3 minutes, 60 °C for 1 minute, and 72 °C for 2 minutes, followed by 30 cycles at 95 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 1 minute and 15 seconds, and 1 cycle at 72 °C for 10 minutes, using a thermal cycler (Eppendorf Mastercycler Gradient, New York, NY). Amplified products were digested at 37 °C (*Sac*I) or 65 °C (*Bsr*I) for 3.0 hours with 5 U of the enzyme. The fragments were separated on a 1.5% agarose gel and visualized under ultraviolet light after staining with ethidium bromide.

Statistical Analysis

All analyses were performed with the SAS statistical analysis package (SAS Institute Inc., Cary, NC). A χ^2 test was performed to assess whether the observed genotype frequencies were in Hardy-Weinberg equilibrium. Differences in allele and genotype frequencies in different cohorts, between men and women, and between BMI classes were assessed by a χ^2 test. Associations between the gene markers and phenotypes in the QFS and HERITAGE studies were tested using the MIXED model procedure for association studies, which takes the nonindependence of family members into account. In this model, data were organized by family identification and family members were assigned a consecutive individual identification within each family. BMI and FM were adjusted for age and sex effects. Plasma lipid and lipoprotein concentrations were adjusted for age and sex plus total FM in some analyses. Abdominal visceral fat area and serum IGF-1 levels were adjusted for total FM, age, and sex. $V\text{O}_{2\text{max}}$ was adjusted for age, sex, and body weight and RQ for FM, FFM, age, and sex. Because of the skewed distributions, abdominal visceral fat area, serum IGF-1, and plasma triglyceride values were log-transformed. BMI, FM, and plasma cholesterol showed skewed distributions in the QFS and were therefore log-transformed (but not in the HERITAGE cohort). In SOS cohorts, BMI showed normal distribution. RQ and high-density lipoprotein-cholesterol in both QFS and HERITAGE cohorts, as well as $V\text{O}_{2\text{max}}$ in the HERITAGE cohort, were normally distributed.

Results

Allele Frequencies of the Variants in Different Cohorts and in Classes of BMI

The allele frequencies for the ghrelin Arg51Gln and preproghrelin Leu72Met polymorphisms in the different cohorts are presented in Table 1. The Gln51 allele was more common in both SOS cohorts ($p = 0.023$ and 0.029 for obese registry and reference populations, respectively) than in QFS or white subjects of HERITAGE. In addition, the Gln allele tended to be more common among men than women in all cohorts except QFS. The Gln allele was not observed among blacks. In HERITAGE, the preproghrelin Met72 carrier status was less frequent in blacks than in whites ($p < 0.0001$). The preproghrelin Leu72Met genotype frequencies were in a Hardy-Weinberg equilibrium in each population.

When all the SOS subjects were pooled ($n = 1437$) and genotype frequencies for the ghrelin and preproghrelin polymorphisms among BMI groups were compared, there were no differences except for one case. The prevalence of the subjects who were Met allele carriers of the Leu72Met polymorphism was higher ($\chi^2 = 3.92$, $p < 0.05$, $df = 1$) in subjects whose BMI was ≤25 kg/m^2 (19.2%) than in those with BMI >25 kg/m^2 (14.8%). When higher cut-off points for BMI classification were used (30 or 35 kg/m^2), no significant differences in carrier status frequencies were observed. Met allele carrier status was not associated with height (data not shown).

Mean Phenotypic Results in Classes of Genotypes or Alleles

The Arg51Gln polymorphism was not associated with any of the phenotypes.

Leu72Met Polymorphism and Obesity-Related Phenotypes and Cardiorespiratory Fitness. The Met72Met genotype was associated with the lowest BMI in QFS (trend) and HERITAGE cohorts ($p = 0.020$) and with the lowest FM in

Table 1. Allele frequencies for the ghrelin/preproghrelin variants in different cohorts

	Allele frequencies			
	Ghrelin/ Arg51Gln		Preproghrelin/ Leu72Met	
	Arg	Gln	Leu	Met
Québec Family Study				
All (n = 784)	99.55	0.45	91.96	8.04
Men (n = 340)	99.56	0.44	92.94	7.06
Women (n = 444)	99.55	0.45	91.20	8.78
HERITAGE Family Study				
All Blacks (n = 276)	100.0	0.00*	98.0	1.99†
Men (n = 91)	100.0	0.00	97.8	2.20
Women (n = 185)	100.0	0.00	98.1	1.89
HERITAGE Family Study				
All Whites (n = 502)	99.20	0.80	92.40	7.57
Men (n = 244)	98.98	1.02	93.00	6.97
Women (n = 258)	99.42	0.58	91.90	8.14
SOS (from obese registry)				
All (n = 741)	98.38	1.62‡	91.97	8.03
Men (n = 387)	97.93	2.07	91.60	8.40
Women (n = 354)	98.87	1.13	92.37	7.63
SOS (from reference population)				
All (n = 701)	98.43	1.57‡	91.30	8.70
Men (n = 304)	98.03	1.97	90.95	9.05
Women (n = 397)	98.74	1.26	91.56	8.44

* $p = 0.036$ and † $p < 0.0001$ between blacks and whites in the HERITAGE Family Study.
‡ $p = 0.023$ between the SOS obese registry population and Québec Family Study. $p = 0.029$ between the SOS reference population and Québec Family Study.
SOS, Swedish Obese Subjects Study.

QFS ($p < 0.001$; Table 2). Black subjects who were Met72 allele carriers also showed lower FM ($p = 0.026$) than noncarriers. In the QFS cohort, the Leu72Leu genotype was associated with the highest FM-adjusted abdominal visceral fat ($p = 0.002$). In Table 3, plasma lipids and RQ by preproghrelin Leu72Met genotypes in the QFS cohort are shown. Total triglycerides were higher in subjects who were homozygotes for the Leu72 allele than in those who were Met72 allele carriers ($p = 0.021$). When adjustments for FM were performed, the differences in plasma lipids became only trends. Furthermore, fasting RQ was highest in Met72 noncarriers ($p = 0.037$) in QFS. The associations of the Leu72Met polymorphism with visceral fat or plasma lipids were not observed in the HERITAGE cohort.

Table 4 shows the associations of preproghrelin Leu72Met polymorphism with cardiorespiratory fitness phenotypes and exercise RQ in HERITAGE. Baseline $V_{O_{2max}}$ was lower in white subjects who were homozygotes compared with those who were heterozygotes for the Leu72 allele ($p = 0.023$ when the genotypes are considered and $p = 0.014$ when the carrier status is considered). Exercise RQ measured during 50 W on the cycle ergometer test was lowest in white Leu72Met heterozygotes ($p = 0.002$ across the genotypes). In the same ethnic group, Leu72 allele homozygotes had the highest RQ at 60% of $V_{O_{2max}}$ ($p = 0.030$ when comparing genotypes).

The black subjects who were heterozygotes for the Leu72 allele had higher ($p = 0.019$) serum IGF-1 levels than homozygotes for the Leu72 allele (Table 5).

Leu72Met Polymorphism and Obesity-Related Comorbidities in SOS Registry Subjects. The prevalence of hypertension, assessed from the SOS patient questionnaires, tended to be lowest in subjects with the genotype Met72Met (Table 6). When the genders were analyzed separately, female Met72 allele carriers had a lower prevalence of hypertension than Met72 allele noncarriers ($p = 0.032$). In addition, the prevalence of stroke and the existence of possible or definite ischemic changes in electrocardiograms tended to be highest in subjects who were Leu72 allele carriers.

Ghrelin Polymorphisms and Plasma Ghrelin Concentrations

Plasma ghrelin levels were analyzed in the Arg51Gln mutation subjects ($n = 7$; plasma not available in one case) and were compared with the levels in age-, sex-, and BMI-matched subjects without the mutation ($n = 14$). In addition, plasma ghrelin levels were available in four preproghrelin Met72Met subjects (missing plasma in three of seven cases). Twelve Leu72Met heterozygotes were chosen from all the heterozygotes so that they all were close to the mean age, BMI, and had the same sex distribution as the Leu72Met group. Thirteen age-, sex-, and BMI-matched Leu72 allele homozygote subjects were also included. Plasma ghrelin levels were lower in the Arg51Gln subjects than in those with the Arg51Arg genotype ($p = 0.007$; Figure 1A). The association remained significant after adjustment for FM ($p < 0.05$). The Met72Met genotype tended to be associated with higher ghrelin concentrations (Figure 1B).

Discussion

In this study, we chose these two preproghrelin exonic variants because they were shown to be associated with obesity in our earlier smaller sample (11). Results of the current study showed that white subjects of the HERITAGE cohort who were homozygotes for the preproghrelin Met72 allele had lower BMI values. Met72 carrier status was also associated with lower FM and higher IGF-1 levels among

Table 2. Obesity-related phenotypes by Leu72Met genotypes in Québec and HERITAGE Family Studies

	Leu72Leu		Leu72Met		Met72Met	
	(Group 1)	***n***	**(Group 2)**	***n***	**(Group 3)**	***n***
BMI* (kg/m^2)						
QFS	24.3 (0.5)	658	24.3 (0.7)	114	23.3 (1.4)	7
HERITAGE						
Blacks	28.1 (0.9)	265	26.7 (1.4)	11	—	
Whites	26.0 (0.4)	433	26.5 (0.7)	62	24.7 (0.5)†	7
Fat mass* (kg)						
QFS	19.5 (1.1)	543	19.9 (1.3)	93	13.7 (1.3)‡	5
HERITAGE						
Blacks	25.1 (1.8)	222	18.4 (2.1)§	8	—	
Whites	21.4 (1.0)	414	20.7 (1.7)	56	22.0 (1.3)	7
CT abdominal Visceral fat¶ (cm^2)						
QFS	89.9 (4.1)**	428	80.3 (5.6)	71	76.6 (4.6)	3
HERITAGE						
Blacks	56.4 (4.0)	218	70.3 (14.5)	8	—	
Whites	73.9 (3.2)	409	68.2 (3.9)	56	81.0 (7.0)	7

Values are means (SE).
* Adjusted for age and sex.
† $p = 0.020$ for trend: $p < 0.01$ between groups 3 and 2, $p < 0.05$ between groups 3 and 1.
‡ $p < 0.001$ for trend: $p < 0.005$ between groups 3 and 2 or 1.
§ $p = 0.026$.
¶ Adjusted for age, sex, and fat mass.
** $p = 0.002$ for trend: $p = 0.007$ between groups 1 and 3, $p = 0.017$ between 1 and 2.
BMI, body mass index; QFS, Québec Family Study; CT, computed tomography.

Table 3. Plasma lipids and fasting respiratory quotient by Leu72Met genotypes in the Québec Family Study

	Leu72Leu		Leu72Met		Met72Met		Met72 carriers	
	(group 1)	***n***	**(group 2)**	***n***	**(group 3)**	***n***	**(groups 2 + 3)**	***n***
Total cholesterol* (mM)	4.71 (0.08)	656	4.74 (0.11)	114	4.91 (0.21)	7	4.76 (0.11)	121
HDL-cholesterol* (mM)	1.24 (0.02)	656	1.28 (0.04)	114	1.27 (0.07)	7	1.28 (0.04)	121
Total triglycerides* (mM)	1.26† (0.05)	656	1.15 (0.05)	114	1.04 (0.14)	7	1.14 (0.05)	121
RQ‡	0.791§ (0.005)	538	0.779 (0.007)	93	0.787 (0.019)	5	0.780 (0.007)	98

Values are means (SE).
* Adjusted for age and sex.
† $p = 0.061$ for trend when genotypes are considered and $p = 0.021$ between group 1 and groups 2 + 3 when the carrier's status is considered.
‡ Adjusted for age, sex, fat mass, and fat-free mass.
§ $p = 0.037$ between group 1 and groups 2 + 3 when the carrier's status is considered.
HDL, high-density lipoprotein; RQ, respiratory quotient.

Table 4. Fitness phenotypes and exercise respiratory quotient by Leu72Met genotypes in the HERITAGE Family Study

	Leu72Leu		Leu72Met		Met72Met		Met72 carriers	
	(group 1)	*n*	(group 2)	*n*	(group 3)	*n*	(groups 2 + 3)	*n*
Baseline VO_{2max} (mL/min)*								
Blacks	2250.2 (38.4)	258	2109.4 (94.1)	11	—		2109.4 (94.1)	11
Whites	2494.3 (33.7)†	426	2647.7 (51.1)	59	2441.0 (112.3)	7	2619.7 (43.3)	66
Baseline maximal power output (W)*								
Blacks	162.9 (3.7)	258	150.0 (7.6)	11	—		150.0 (7.6)	11
Whites	186.2 (3.7)	426	195.7 (4.3)	59	187.2 (14.5)	7	194.6 (3.8)	66
RQ (during 50 W)‡								
Blacks	0.943 (0.007)	220	0.947 (0.016)	8	—		0.947 (0.016)	8
Whites	0.918 (0.004)	405	0.905 (0.006)§	56	0.925 (0.007)	7	0.908 (0.006)	63
RQ (at 60% VO_{2max})‡								
Blacks	0.971 (0.007)	222	0.978 (0.017)	8	—		0.978 (0.017)	8
Whites	0.967 (0.004)¶	412	0.950 (0.006)	56	0.961 (0.006)	7	0.951 (0.005)	63

Values are means (SE).
* Adjusted for age, sex, and body weight.
† $p = 0.023$ for trend when genotypes are considered. $p < 0.01$ between groups 1 and 2. When the carrier's status is considered. $p = 0.014$ between group 1 and groups 2 and 3.
‡ Adjusted for age, sex, fat mass, and fat-free mass.
§ $p = 0.002$ for trend when genotypes are considered. $p < 0.01$ between groups 2 and 3, $p < 0.05$ between groups 2 and 1.
¶ $p = 0.030$ for trend when genotypes are considered. $p < 0.01$ between groups 1 and 2. When the carrier's status is considered, $p < 0.01$ between group 1 and groups 2 + 3.
RQ, respiratory quotient.

blacks. In the QFS cohort, Met72 carriers had lower values for FM, abdominal visceral fat, and fasting RQ. White subjects of the HERITAGE cohort who were carriers of Met72 also showed lower exercise RQ and higher cardiorespiratory fitness. Furthermore, the prevalence of Met allele carriers was higher in SOS subjects whose BMI was ≤ 25 kg/m^2 than in those with BMI >25 kg/m^2. SOS female subjects from the obese registry, who were Met72 allele carriers, had a lower prevalence of hypertension than Met72 allele noncarriers.

Table 5. Mean serum IGF-1 levels (ng/mL) by Leu72Met polymorphism in the HERITAGE Family Study

	Leu72Leu		Leu72Met		Met72Met		Met72 carriers	
	(group 1)	*n*	(group 2)	*n*	(group 3)	*n*	(groups 2 + 3)	*n*
Blacks	114.7 (7.0)	212	161.8* (20.7)	8	—	0	161.8 (20.7)	8
Whites	105.8 (4.4)	411	113.2 (6.9)	55	110.9 (18.5)	7	112.9 (6.3)	62

* $p = 0.019$.
Values are means (SE).
Adjusted for age, sex, and fat mass.
IGF-1, insulin-like growth factor-1.

Table 6. Obesity-related comorbidities by Leu72Met polymorphism in SOS obese registry subjects

	Leu72Leu	*n*	Leu72Met	*n*	Met72Met	*n*
Hypertension*						
All	27.3%	626	25.0%	104	14.3%	7
Men	30.5%	325	35.0%	60	50%	2
Women	23.9%	301	11.4%	44	0%†	5
Diabetes mellitus*						
All	7.0%	626	8.6%	104	0%	7
Men	7.4%	325	11.7%	60	0%	2
Women	6.6%	301	4.5%	44	0%	5
Stroke*						
All	1.3%	626	0%	104	0%	7
Men	1.8%	325	0%	60	0%	2
Women	0.7%	301	0%	44	0%	5
Signs of ischemia in ECG						
All	5.7%	626	2.9%	104	0%	7
Men	7.3%	325	5.0%	60	0%	2
Women	4.0%	301	0%	44	0%	5

* According to the patient questionnaires.
† $p = 0.032$ between Met72 carriers and noncarriers.
SOS, Swedish Obese Subjects Study; ECG, electrocardiogram.

In the QFS cohort, preproghrelin Leu72 allele homozygote subjects had more abdominal visceral fat. Their plasma triglycerides and RQ were higher than those of Met72 allele carriers. The associations with plasma lipids in QFS were not significant after adjustment for total FM, which indicates that the association of Leu72Met polymorphism with plasma lipids was mediated through adiposity. An association between the preproghrelin Leu72Leu genotype and a

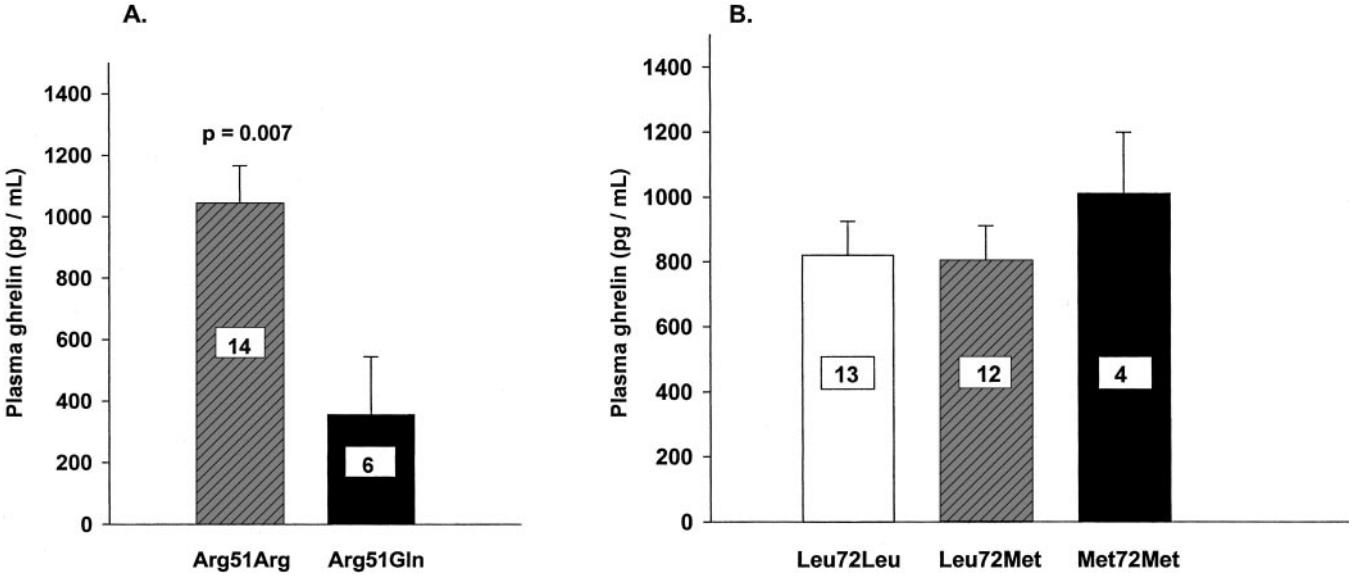

Figure 1: Plasma ghrelin levels in relation to ghrelin Arg51Gln (A) and preproghrelin Leu72Met (B) genotypes.

high exercise RQ, but not visceral fat, was also observed in the HERITAGE white population. A high RQ has been associated with the risk of gaining weight because more fat is stored instead of being oxidized (28), although the matter is still controversial (29), and its role in the tendency to store more fat into abdominal depot is largely unknown. Interestingly, ghrelin induces a positive energy balance in rodents by decreasing fat oxidation (7), although this has not yet been studied in humans and the effects of preproghrelin are unknown to date.

The mutation at codon 72 of the preproghrelin gene (Leu72Met) is outside the coding region of the mature ghrelin (10). Whether preproghrelin yields other products with physiological effects different from those of ghrelin is as of yet unknown, although it is known that motilin-related peptides and their precursors share close homology with preproghrelin (30). One might speculate that preproghrelin undergoes similar processing in the stomach as the pro-opiomelanocortin does in the brain, yielding different products dependent on the stimulus leading to their production (31,32). The sequence variation in the preproghrelin gene could then theoretically change the structure of one or more of these products. The latter could have functional consequences with more specific binding for the putative ghrelin receptors (growth-hormone secretagogue receptor 1a) that are responsible for stimulation of growth hormone secretion. It is important to note that in this study, black subjects who were preproghrelin Met72 carriers had lower FM and higher plasma IGF-1 levels that, in most conditions, reflect growth hormone levels quite well (33). In addition, recent investigations have suggested that accumulation of fat in the abdominal depot is associated with endocrine disturbances that include a blunted secretion of growth hormone (34). These endocrine perturbations may also have causal effects on abdominal visceral fat accumulation (34). Therefore, low growth hormone levels among white subjects who were Leu72 allele homozygotes could theoretically also explain their tendency to gain more visceral fat, although IGF-1 levels were not assayed in the QFS. Plasma ghrelin levels were measured in a subgroup of QFS subjects, and Met72 allele homozygotes tended to have higher ghrelin levels. Leu72Leu white subjects in HERITAGE also had lower $V\mathrm{O}_{2\mathrm{max}}$. Some studies have shown that increased growth hormone pulsatility was associated with a higher peak oxygen uptake (35). However, the Leu72Met polymorphism was not associated with IGF-1 levels in whites.

Whether Met72 carrier status makes ghrelin more or less potent is, however, unknown. If the latter is true, then a decreased influence of ghrelin on hypothalamic neuropeptide networks (through a putative ghrelin receptor subtype that is specific for effects on appetite or energy homeostasis) would lead to an imbalance between leptin and ghrelin at those neurons and finally to an increased fat oxidation and decreased adiposity.

A mutation at amino acid position 51 of the preproghrelin leads to a replacement of arginine by glutamine in codon 28, the last codon of the mature ghrelin product. Amino acid Arg51 is a target site for the action of endoproteases (36) and for the proteolytic cleavage of 66 carboxy-terminal amino acids to produce mature ghrelin. The Arg51Gln mutation disrupts this recognition site. Interestingly, the mutation seems to be associated with lower plasma ghrelin levels, and the association remained significant after adjustment for FM. However, it does not seem to have overt clinical effects, even though it was more common in SOS than QFS or HERITAGE cohorts and more in whites than blacks. Thus, the results of this study are in disagreement with those of our previously published smaller study—in which an association between the Arg51Gln mutation and obesity was suggested (11). Furthermore, the observation that the preproghrelin Met72 carrier status might predispose to obesity was not confirmed in the current study. Acylation of the hydroxyl group of serine at position 3 by *n*-octanoyl acid is necessary for the binding of ghrelin to the growth-hormone secretagogue receptor (1). A recent structure-function study suggests that short peptides encompassing the first four or five residues of ghrelin and including the Ser3 were found to be as active as full-length ghrelin (37).

In conclusion, the ghrelin Arg51Gln mutation was associated with plasma ghrelin levels but not with obesity. The preproghrelin Met72Met genotype was associated with lower BMI, FM, and abdominal visceral fat in whites. Met72 carriers also had lower FM and higher IGF-1 levels in blacks. White subjects who were Met72 carriers had lower resting and exercise RQ, as well as higher cardiorespiratory fitness. Furthermore, the prevalence of the Met72 allele was higher in Swedish lean than in overweight or obese subjects. SOS female obese registry subjects who were Met72 allele carriers had a lower prevalence of previously known hypertension than Met72 allele noncarriers. Thus, the preproghrelin Met72 carrier status seems to be protective against fat accumulation and associated metabolic comorbidities. However, the associations were not very strong and were for low-frequency alleles. The mechanisms for these associations are unknown. We speculate, that both decreased ghrelin-induced signaling at the hypothalamic neuroendocrine network regulating energy balance as well as increased stimulation of the somatotropic axis (GH-IGF-1 axis) through ghrelin receptor activation among Met72 allele carriers could potentially be involved (38).

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