Original Article

Variants within the Ghrelin Gene – Association with HDL-Cholesterol, but Not with Body Mass Index

(ghrelin / polymorphism / BMI / WHR / HDL-cholesterol)

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Abstract. Ghrelin is a hormone which influences eating habits, the amount of food ingested and the body's energy balance. We examined whether genetic variants in the ghrelin gene are associated with BMI, WHR and plasma lipid levels. We have evaluated the influence of ghrelin polymorphisms (Arg51>Gln, Leu72>Met and Gln90>Leu) on BMI, WHR, and plasma lipid levels in 1,191 males and 1,368 females representatively selected from the Czech population. Anthropometrical and biochemical parameters were analysed in two different years. In the entire population, we have detected 4.8% of carriers of the Gln51 allele, 14.2% carriers of the Met72 allele, and 10.9% of the Leu90 allele. Frequencies did not differ between males and females and alleles were not in linkage disequilibrium. BMI or WHR were not influenced by variants in the ghrelin gene. The ghrelin variant Leu72>Met was associated with elevated levels of plasma HDL-cholesterol. Compared to Leu/Leu homozygotes, the Met carriers had lower HDL-cholesterol concentrations in males (1.18 ± 0.29 mmol/l vs. 1.24 ± 0.35 mmol/l, P = 0.01) as well as in females (1.45 ± 0.35 mmol/l vs. 1.51 ± 0.38 mmol/l, P = 0.01). The other lipid parameters (total cholesterol and triglycerides) were not associated with this variant. There were no associations between other ghrelin variants (Arg51>Gln and Gln90>Leu) and analysed biochemical parameters. We conclude that in the Caucasian population, variations in the ghrelin gene could play a role in genetic determination of plasma levels of HDL-cholesterol, but they have no effect on BMI or WHR.

Obesity is one of the major health problems in most of the population around the world and a large proportion of obese individuals die from overweight-related complications. The incidence of obesity is commonly in excess of 15% in various populations, but reaching about 30% in middle and east European countries (data from the MONICA project).

The development of overweight is no doubt influenced by a variety of external factors (most importantly, by excess energy intake and lack of exercise, but also, for example, being a member of a lower social class), but genetic factors also play an important role in overweight development (body mass index [BMI] determination). It is estimated that the heritability of obesity is between 30% and 70%.

Among the obesity-related candidate genes, ghrelin is one of the most recently described.

Ghrelin is an endogenous peptide, first described by Kojima et al. (1999). Its active form is composed of 28 amino acids (ghrelin precursor – preproghrelin – has 117 amino acids) and circulates in plasma at a concentration ~ 800 – 1000 pg/ml.

The major source of ghrelin secretion is stomach, but other organs (hypothalamus, hypophysis, duodenum, kidney) also produce substantial amounts of the hormone (Kojima et al., 1999; Ariyasu et al., 2001). Ghrelin is a ligand for the growth hormone secretagogue receptor (Kojima et al., 1999), which stimulates growth hormone release. The role of ghrelin in obesity induction has been proved in animal experiments. Administration of ghrelin in animals leads to an increased food intake, decreased fat utilization and decreased energy expenditure (for review see Ukkola and Pöykkö, 2002).

In humans, the plasma levels of ghrelin are inversely correlated with BMI, and the highest plasma levels were found in patients with anorexia and the lowest in obese subjects (Tschöp et al., 2001; Shiiya et al., 2002). Plasma levels of ghrelin are elevated in preprandial state (Cummings et al., 2001) and ghrelin, injected to healthy volunteers, induced sensation of hunger (Wren et al., 2001).

In the human gene for ghrelin, one common mutation (2-bp deletion at codon 34) (Hinney et al., 2002) and three polymorphisms have been described so far (Uk-

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Abbreviations: BMI – body mass index; HDL – high-density lipoprotein; LDL – low-density lipoprotein; PCR – polymerase chain reaction; WHR – waist hip ratio.

kola et al., 2001; Hinney et al., 2002). The base change G152>A results in amino acid exchange Arg51>Gln, exchange 214C>A in Leu72>Met polymorphism, and the last variant represents A271>T transversion leading to Gln90>Leu exchange. Variants Leu72>Met and Gln90>Leu have been suggested, but not unequivocally confirmed, to play a role in the genetic determination of body weight. With one exception, so far analysed populations comprise only 44–300 individuals of both sexes. We have analysed the putative associations between anthropometrical and lipid parameters and all three ghrelin variants in a large ethnically homogenous population of 2 500 Caucasians.

Material and Methods

Study population

The 2,559 unrelated Caucasians (1,191 males and 1,368 females) included in this study represented a 3-year cohort of the selected 1% Czech population sample from nine districts. The individuals were recruited in 1997–1998 and reinvited in 2000–2001 according to the protocol used for the MONICA study [*Multinational monitoring of trends and determinants in cardiovascular diseases: "MONICA Project"*. Manual of operations WHO/MNC 82.2, Nov-1983.]. Anthropometrical and lipid parameters are available for both years. Written informed consent was obtained from the study participants, and the local ethic committee approved the design of the study. Basic characteristics of the study participants are summarized in Table 1.

Genotype analysis

Three ml of blood collected into EDTA tubes for DNA isolation were stored at -20°C. DNA was isolated by the standard salting-out method (Miller et al., 1988). Three ghrelin variants were analysed by polymerase chain reaction (PCR) and restriction analysis. PCR device DYAD was used to perform the PCR reaction. DNA was amplified using primers 5'aggacctgggggcttagagt and 5'gctgccacagaagcataaaa (for variants at amino acid positions 51 and 72) and 5'ttgagatggggatgggcatgacctctg and 5'ccccagggcctggctgtgctgcttgta (for variant at amino acid position 90). Ten µl of PCR product were digested in a total volume of 25 µl with the appropriate restriction enzyme (position 51 – *Sac*I, position 72 – *Bse*N1, and position 90 – *Bsr*G1) at 37°C overnight in the buffer provided by the manufacturer. Restriction fragments were separated on 12% polyacrylamide gel using the MADGE technique (Day and Humphries, 1994).

Analytical methods

Blood pressure and anthropometric parameters were measured by a trained nurse and the BMI was calculated as weight in kilograms divided by the height (in meters) squared. The lipoprotein parameters were measured in the WHO Regional Lipid Reference Centre, Institute for Clinical and Experimental Medicine, Prague in a Hoffman-La Roche (Basel, Switzerland) COBAS MIRA autoanalyser. Serum triglycerides and total cholesterol levels were determined using conventional enzymatic methods with reagents from Hoffmann-La Roche (kits No. 0722138 and 0715166). Serum HDL-cholesterol was analysed by the phosphothungstate/$MgCl_2$ precipitation procedure (Hoffmann-La Roche kit No. 0720674).

Statistical analysis

Statistical analysis was performed using the ANOVA test. Triglycerides were logarithmically transformed before the analysis to obtain the normal distribution of data. As the numbers of less common homozygotes were very low (Table 2), rare homozygotes were pooled for statistical analysis with heterozygotes.

Results

Individual ghrelin alleles are not in linkage disequilibrium and all three ghrelin polymorphisms are in Hardy-Weinberg equilibrium. The allelic frequencies of different variants did not differ between males and females (Table 2). The allelic frequencies of all three analysed variants were similar to others in so far that the analysed populations were of the same ethnicity.

Table 1. Basic characteristics of the individuals involved in the study (control data are from 2001, given as mean ± SD)

	Males	**Females**
N	1191	1368
Age (years)	49.2 ± 10.8	48.8 ± 10.6
BMI (kg/m^2)	28.2 ± 4.0	27.6 ± 5.5
WHR	0.929 ± 0.064	0.810 ± 0.072
Cholesterol (mmol/l)	5.75 ± 1.06	5.80 ± 1.15
Triglycerides (mmol/l)	1.98 ± 1.28	1.46 ± 0.85
HDL-cholesterol (mmol/l)	1.26 ± 0.33	1.50 ± 0.36
Diabetes (N/%)	72/6.0	60/4.4
Hypertension (N/%)	489/41.1	457/33.4
Smoking prevalence (N/%)	389/32.7	348/25.4

Table 2. Frequencies of the ghrelin variants in Czech population

	Males		Females	
	N	%	N	%
	Arg 51 > Gln			
Arg/Arg	1134	95.3	1301	95.2
Arg/Gln	56	4.7	64	4.7
Gln/Gln	0	0	2	0.1
	Leu 72 > Met			
Leu/Leu	1021	85.9	1168	85.6
Leu/Met	164	13.8	189	13.9
Met/Met	4	0.3	7	0.5
	Leu90 > Gln			
Gln/Gln	1052	88.6	1219	89.4
Gln/Leu	129	10.9	137	10.1
Leu/Leu	6	0.5	7	0.5

Arg51>Gln variant

We have detected only two Gln51Gln homozygotes (both females) and 4.7% of Arg/Gln heterozygotes both in males and females.

This variant was not significantly associated with BMI or WHR either in males or in females (Table 3). The lipid parameters were also independent of this variant.

Leu72>Met variant

Also in this case the homozygosity of the less common allele was rare in the population; we have detected only 0.5% of the homozygotes and ~ 14% of the carriers of the Met72 allele.

This variant had no effect on BMI or WHR (Table 3).

We have detected a significant association between the Leu72>Met variant and plasma levels of HDL-cholesterol. Common Leu/Leu homozygotes had higher HDL-cholesterol than carriers of the less common allele Met72 (Table 4). This association was detected both in males (1.24 ± 0.35 mmol/l vs. 1.18 ± 0.30 mmol/l, $P < 0.01$) and females (1.51 ± 0.38 mmol/l vs. 1.45 ± 0.35 mmol/l, $P < 0.01$). Other lipid parameters (total cholesterol, triglycerides) were not influenced by this gene variant.

Gln90>Leu variant

A half percent of the population were Leu90Leu homozygotes and slightly more than 10% were carriers of one Leu90 allele.

This ghrelin variant had no effect on plasma lipids, BMI or WHR either in males or in females (Table 3).

Haplotype analysis

Out of the 27 possible haplotypes, we have detected altogether 11 present in our population. As expected, the most common haplotype was ArgArg51/LeuLeu72/GlnGln90, and it was found at the frequency ~ 70% in the population. Analysis of associations for individual haplotypes did not reveal any significant associations with anthropometrical or lipid parameters (data not shown).

Table 3. Ghrelin variants and BMI (kg/m²) and WHR

	Males		
	N	BMI	WHR
Arg/Arg	1134	27.9 ± 3.9	0.928 ± 0.066
+ Gln	56	28.0 ± 3.3	0.932 ± 0.058
P		n.s.	n.s.
Leu/Leu	1021	28.1 ± 4.0	0.929 ± 0.071
+ Met	168	28.5 ± 3.8	0.936 ± 0.071
P		n.s.	n.s.
Gln/Gln	1052	27.9 ± 3.9	0.928 ± 0.066
+ Leu	135	28.0 ± 4.1	0.929 ± 0.063
P		n.s.	n.s.
	Females		
	N	BMI	WHR
Arg/Arg	1301	27.2 ± 5.4	0.810 ± 0.072
+ Gln	66	28.4 ± 5.9	0.815 ± 0.082
P		n.s.	n.s.
Leu/Leu	1168	27.5 ± 5.6	0.810 ± 0.076
+ Met	196	27.8 ± 5.2	0.813 ± 0.072
P		n.s.	n.s.
Gln/Gln	1219	27.3 ± 5.5	0.810 ± 0.072
+ Leu	144	27.0 ± 4.9	0.817 ± 0.070
P		n.s.	n.s.

Discussion

Although it is clear that abundant energy intake is the most important risk factor for obesity development, studies from previous years suggest that genetic predispositions will also play a significant role in determination of body weight.

In the Czech population, obesity is one of the most serious health risks. According to the MONICA study in 2000, 29.5% and 28.1% of men and women, respectively, are obese while overweight afflicts 45.9% of men

Table 4. Leu72>Met variant in ghrelin and plasma levels of HDL-cholesterol (mmol/l)

	Males		Females	
	N	HDL-cholesterol	N	HDL-cholesterol
Leu/Leu	1021	1.24 ± 0.35	1168	1.51 ± 0.38
+ Met	168	1.18 ± 0.29	196	1.45 ± 0.35
P		0.01		0.01

and 32.4% of women (Blazejova et al., 2000). It is well detectable by means of BMI in our study, which is both for males and females more than 27 kg/m^2.

Despite intensive analysis in previous years, the genetic determination of obesity in a population is not yet understood. Variants in genes for leptin, tissue-specific uncoupling protein 1, glucocorticoid receptor, adiponectin, perilipin, or PPAR-γ2 are suggested to have important roles in genetic determination of BMI development (Clement, 2006).

Ghrelin, first mentioned at the end of the last century, is a biologically active gastrointestinal peptide that affects eating habits, regulates body energy balance, and has an effect on the sleeping pattern. Thus, it is a good candidate for which one would suppose an important role in BMI determination. The significance of ghrelin in body weight determination was proved in animal and human experimental studies. In the human gene for ghrelin, three common variants have effects on the amino acid sequence of the preprotein (comprising only 117 amino acids), and such polymorphism density is not commonly observed.

In our large adult population, with high overweight frequency, we have failed to detect any significant associations between the variants in the ghrelin gene and obesity-related anthropometrical measures. Neither individual polymorphisms, nor different haplotypes were associated with BMI or WHR.

Variants in the ghrelin/preproghrelin gene (Arg51/Gln and Leu72/Met) were first described by Ukkola et al. (2001). Their data indicated that the Arg51Gln variant could be associated with the aetiology of obesity. The same authors (Ukkola et al., 2002) later published the results of three different studies (Quebec Family Study, HERITAGE Family Study and Swedish Obese Subject study), and although the results were not homogenous, the Met72 allele was suggested to be associated with favourable obesity-related phenotypes (lower BMI and lower fat mass [in Africans only]). Further, association between BMI and ghrelin Leu72Met variant was described in a group of obese tall children (Korbonits et al., 2002). Finally, Miraglia de Giudice et al. (2004) have reported that individuals with at least one Met72 allele became obese significantly earlier than homozygotes for the common Leu72 allele. Gln90Leu exchange (Hinney et al., 2002) had no clear effect on BMI.

Is it possible that in the Czech population (with mean BMI over 27 kg/m^2), where the energy intake is too high and physical activity too low in the majority of the subjects, the power of the genetic predisposition (detectable before adulthood) could be hidden by these negative external factors.

As dietary habits (in this case, however, probably not only the amount of consumed food, but predominantly the food composition) have an effect on plasma lipid levels, we have also analysed an association between ghrelin variants and plasma lipid parameters. We have detected an association between Leu72>Met variant and plasma HDL-cholesterol levels. There is no plausible explanation for this association, and the role of ghrelin in HDL particle metabolism is unclear. Nevertheless, as the number of analysed individuals was over one thousand both for the males and females, the association was not sex-specific, and we have detected a similar association both in the 1997–1998 and in 2000–2001 surveys, we believe that the detected association represents real and not only false-positive results. This is further supported by the fact that a similar association was described in individuals of Korean ethnicity (Choi et al., 2006). The authors have described an association between plasma HDL-cholesterol levels and Leu72>Met and G-1062>C variants.

So far, no functional studies of individual ghrelin variants have been performed, but it is known that the Met72Met homozygotes have higher plasma levels of ghrelin. As we (and others: Choi et al., 2006) have detected elevated levels of HDL-cholesterol in Leu72Leu homozygotes, we can speculate that a lower ghrelin concentration will have an effect on plasma HDL-cholesterol concentration, but the mechanism is completely unclear.

We conclude that there was no association between any of the ghrelin polymorphisms analysed (Arg51>Gln, Leu72>Met and Gln90>Leu) and BMI or WHR in a large Caucasian population. Nevertheless, both in males and females, we have detected an association between plasma levels of HDL-cholesterol and the Leu72>Met variant in the ghrelin gene.

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