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Genetics

Association Between Ghrelin Gene Variations and Blood Pressure in Subjects With Impaired Glucose Tolerance

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Background: Ghrelin is a gut–brain hormone, which stimulates food intake and controls energy balance. Recently, it has been shown that ghrelin may also play a role in the regulation of blood pressure (BP) by acting at the sympathetic nervous system. In the present study we genotyped six variants of the ghrelin gene and its promoter, and tested whether these single nucleotide polymorphisms (SNPs) were associated with BP levels in participants of the Finnish Diabetes Prevention Study.

Methods: The Finnish Diabetes Prevention Study was a longitudinal study where 522 subjects with impaired glucose tolerance were randomized into either an intervention or control group. DNA was available from 507 subjects (mean body mass index [BMI] 31.2 ± 4.5 kg/m^2, age 55 ± 7 years). All six SNPs were screened by the restriction fragment length polymorphism method.

Results: Subjects with the most common genotype combination of the following four SNPs, -604G/A, -501A/C, Leu72Met, and Gln90Leu, had the lowest systolic (131 ± 11 *v* 137 ± 13 mm Hg, $P = .003$) and diastolic BP levels (79 ± 7 *v* 83 ± 7 mm Hg, $P = .004$) at the baseline of the study and during 3 years of follow-up compared to all other genotypes. Adjustments for age, gender, antihypertensive medication, BMI, waist circumference, and alcohol intake did not change this association.

Conclusions: Several ghrelin gene variations were associated with BP levels in subjects with impaired glucose tolerance. Am J Hypertens 2006;19:920–926

Key Words: Ghrelin, SNPs, variation, blood pressure, hypertension, impaired glucose tolerance.

Ghrelin, a 28-amino acid peptide, was originally isolated from the rat stomach as an endogenous ligand for the growth hormone secretagogue receptor (GHSR).[1] Ghrelin strongly stimulates the release of growth hormone (GH) from the pituitary gland[2,3] and acts as a gut–brain regulatory peptide stimulating food intake and controlling energy balance.[4–6] Recently, ghrelin has been shown to play a role in central cardiovascular and sympathetic regulation.[7–11] Ghrelin has a vasodilatory effect in humans,[7] and intravenous injection of human ghrelin has been shown to elicit a decrease in blood pressure (BP) without an increase in heart rate in healthy men.[8] Intracerebroventricular administration of ghrelin has been shown to decrease arterial pressure in conscious rabbits, suggesting that ghrelin may be involved in cardiovascular and sympathetic regulation in the central nervous system.[9]

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Lin et al[12] suggested that suppression of the sympathetic nervous system is the primary mechanism of a decrease in arterial pressure and that ghrelin acts at the neurons of the nucleus of the solitary tract to decrease arterial pressure in rats. Tsubota et al[13] have shown that both ghrelin and des-acyl ghrelin elicit hypotensive responses when microinjected into nucleus tractus solitarii of rats. The nucleus of the solitary tract, where baroreceptor and chemoreceptor afferents terminate, is one of the most important brain regions to regulate BP and the sympathetic nervous system.[10,14] Cardiovascular and sympathetic effects of ghrelin are most probably attributable to ghrelin itself and therefore they are GH independent.[10,15,16] Ghrelin peptide and mRNA have been detected in a variety of tissues including the heart and blood vessels, where GHSR mRNA is also expressed.[8,17] Cardiovascular effects of ghrelin are probably mediated by multiple receptors, some of them still to be identified.[13,18,19]

The human ghrelin gene is located at the chromosomal locus 3p26-p25, and the prepro-hormone is encoded by five exons.[20] Several single nucleotide polymorphisms (SNPs) in the coding region for the prepro-ghrelin have been described, but data on humans linking genetic variations in the ghrelin gene to BP levels or hypertension are sparse. The Gln51 allele of the Arg51Gln SNP has been found to be a risk factor for hypertension,[21] but data concerning the Leu72Met SNP are inconsistent.[22–24] To date, there is only one report of a polymorphism in the promoter region of the ghrelin gene.[25]

In the present study we genotyped six variants of the ghrelin gene, two of which have been shown to be involved in BP regulation,[21,23] and tested whether these SNPs were associated with BP levels or the presence of hypertension in the participants of the Finnish Diabetes Prevention Study.

Methods

Subjects and Study Design

The Finnish Diabetes Prevention Study was an intervention study carried out in five centers. The main aim of the study was to assess the efficacy of an intensive individually designed diet and exercise program to prevent or delay the onset of type 2 diabetes in subjects with impaired glucose tolerance (IGT). The study design, subjects, inclusion and exclusion criteria, and intervention program have been described earlier in detail.[26,27] In brief, 522 middle-aged (40 to 65 years) and overweight (body mass index [BMI] $\geq$25 kg/m^2) subjects with IGT participated in the study. Impaired glucose tolerance was defined as a 2-h plasma glucose concentration of 7.8 to 11.0 mmol/L (oral glucose tolerance test [OGTT] 75 g) with a fasting plasma glucose concentration of less than 7.8 mmol/L.[28] The subjects enrolled in the study were randomly assigned to one of the two treatment modalities, the intervention group or the conventional care control group. The study protocol was approved by the Ethics Committee of the National Public Health Institute in Helsinki, Finland, and conducted in accordance with the guidelines proposed in the Declaration of Helsinki. All participants gave written informed consent.

Blood Pressure Phenotype Determination

Physical examination and measurements of clinical and biochemical characteristics were performed at baseline and at each annual follow-up visit. Blood pressure was measured by trained study nurses using a standard sphygmomanometer twice on the right arm after 10 min of rest with the subject in a sitting position. The mean of systolic and diastolic BP was calculated from two measurements obtained. Hypertension was defined as the mean systolic BP of $\geq$140 mm Hg or diastolic BP of $\geq$90 mm Hg, or taking antihypertensive medication.[29,30] Subjects were on different antihypertensive drug treatments during the study period including angiotensin-converting enzyme (ACE) inhibitors, calcium channel blockers, general β-blockers and β_1-specific blockers, α-blockers, angiotensin receptor-blocking agents, diuretics, and antihypertensive agents with central mechanism of action, and combination therapies. Altogether 35.5% of the participants had antihypertensive drug treatment in the beginning of the study.

Assessment of Ghrelin Gene Variation Genotypes

The DNA samples were available from 507 of the 522 subjects. The six SNPs were detected by polymerase chain reaction-based–restriction fragment length polymorphism analysis (PCR-RFLP). The following primers were used in this study: 1) for -604G/A: forward 5′-CACAGCAAC-AAAGCTGCACC-3′ and reverse 5′-AAGTCCAGCCAG-AGCATGCC-3′; 2) for -501A/C and -473G/A: forward 5′-AGAACAAACGCCAGTCATCC-3′, reverse 5′-GTC-TTCCAGCCAGACAGTCC-3′: 3) for Arg51Gln and Leu72Met: forward 5′-GCTGGGCTCCTACCTGAGC-3′ and reverse 5′-GGACCCTGTTCACTGCCAC-3′; 4) for Gln90Leu: forward 5′-GAGGTGTCACTCAGCAGTCC-3′ and reverse 5′-TCTTCTTCTTCAGGGCCTGGCTGT-GCTGCTAGTAC-3′ (mismatch nucleotide introduced to the primer is underlined) (TAG Copenhagen A/S, Copenhagen, Denmark). The genomic DNA was amplified by PCR followed by digestion with specific restriction enzymes (Fermentas, Tamro Medlab OY, Vantaa, Finland) for 3 h: 1) *Dra*I at 37°C for -604G/A, 2) *Mwo*I at 37°C for -501A/C, 3) *Fok*I at 55°C for -473G/A, 4) *Sac*I at 37°C for Arg51Gln, 5) *Bsr*I at 37°C for Leu72Met, and 6) *Sca*I at 37°C for Gln90Leu. The fragments were separated on an agarose gel and visualized under ultraviolet light after staining with ethidium bromide (Fig. 1).

Originally, genetic variations in the ghrelin gene were determined by direct sequencing of the coding region and a part of the promoter area (1 kb upstream of the transcription start site) in 35 massively obese Finnish subjects.[31]

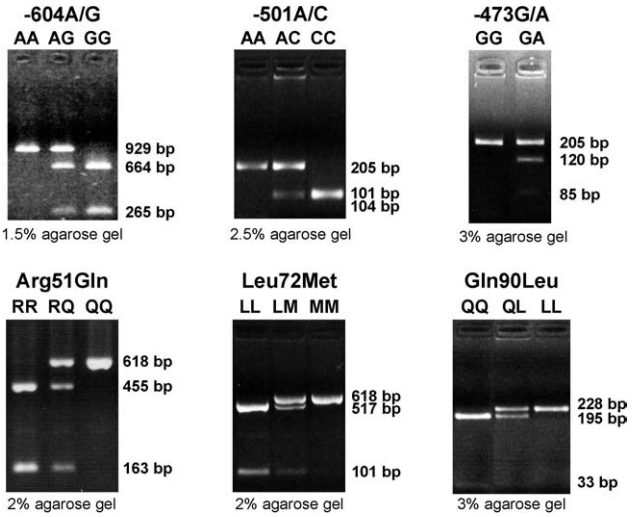


FIG. 1. Restriction fragment length polymorphism (RFLP) analysis of the six SNPs in the ghrelin gene and its promoter. RFLP analysis of the six SNPs was performed with following restriction enzymes: *Dra*I for -604G/A, *Mwo*I for -501A/C, *Fok*I for -473G/A, *Sac*I for Arg51Gln, *Bsr*I for Leu72Met, and *Sca*I for Gln90Leu. The fragments were separated on an agarose gel and visualized under ultraviolet light after staining with ethidium bromide. In the gel pictures the first lane always represents the wild-type homozygotes, the second lane the heterozygotes, and the third lane the mutated homozygotes. For the three SNPs in the prepro-ghrelin protein (Arg51Gln, Leu72Met, and Gln90Leu) the lanes are named with one-letter amino acid codes: R = Arg, Q = Gln, L = Leu, M = Met. Representative gels are shown.

Statistical Analysis

Analyses of genotype combinations were conducted with the four most common SNPs (-604G/A, -501A/C, Leu72Met, and Gln90Leu). Genotype combinations were formed with these four SNPs by combining the wild-type homozygotes (denoted by 0) and the mutated homozygotes (denoted by 2) for each subject.

The significance of differences in genotype and allele frequencies were analyzed using a χ^2 test and a two-sided Fisher's exact test, respectively. Normal distribution was tested with the Kolmogorov-Smirnov (Lilliefors) test. To normalize the skewed distributions, logarithmic transformations or reciprocal transformations were applied when needed. The differences in continuous variables between genotype combination groups at baseline were evaluated with the general linear model (GLM) for the univariate analysis of variance (ANOVA). Biochemical measurements were adjusted for age, gender, and BMI as main effects and weight-related variables for age and gender only. When variables could not be transformed to be normally distributed, Kruskal-Wallis test was used.

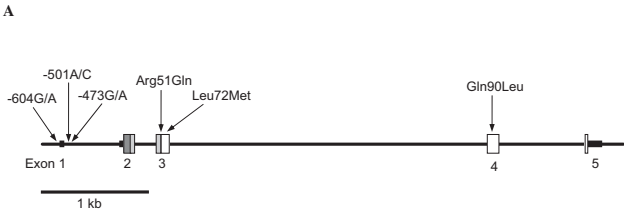
To analyze differences between the genotype combination groups in systolic and diastolic BP levels of four consecutive yearly measurements, linear mixed models analysis for repeated measures data was applied. Categorical variables were genotype, gender, study group, as well as the time-dependent variable antihypertensive medication. Continuous covariates included age at baseline and the time-dependent covariates BMI, waist circumference, and alcohol intake. A final model was built manually with a backward selection procedure. With the linear mixed models procedure covariates can be specifically included for each year separately (ie, changes in antihypertensive drug treatment during the four measurements were taken into account for each individual).

A χ^2 test was used for comparison of categorical variables. Logistic regression analysis was used to determine the risk of hypertension in different genotype combination groups. A P value of .05 or lower was considered statistically significant. Data are given as means ± SD, unless otherwise stated. All data were analyzed using SPSS for Windows 11.5.1 (SPSS Inc., Chicago, IL).

Linkage disequilibrium statistics were calculated and haplotype blocks were visualized by using the Haploview software.[32]

Results

We identified five previously known SNPs, -604G/A (rs27647), -501A/C (rs26802) in the promoter region, Arg51Gln (to date no rs number assigned), Leu72Met (rs696217), and Gln90Leu (rs4684677) in the coding region, as well as one novel variation, -473G/A, in the promoter region of the ghrelin gene and genotyped these in the subjects of the Finnish Diabetes Prevention Study



B

Standardized pairwise linkage disequilibria

		D'				
	SNPs	-604G/A	-501A/C	-473G/A	Arg51Gln	Leu72Met	Gln90Leu
	-604G/A	-	0.674	1.000	1.000	1.000	1.000
	-501A/C	0.104	-	1.000	1.000	0.516	0.897
	-473G/A	0.020	0.005	-	0.026	1.000	1.000
r^2	Arg51Gln	0.019	0.011	0.000	-	1.000	0.786
	Leu72Met	0.090	0.105	0.002	0.005	-	0.875
	Gln90Leu	0.103	0.051	0.002	0.003	0.019	-

FIG. 2. Polymorphic sites in the 5′ region and the coding sequence of the ghrelin gene and their pairwise linkage disequilibria. (**A**) Nucleotide substitutions for three promoter polymorphisms relative to the transcription start site. The Arg51Gln SNP is located in the last codon of the mature ghrelin protein and disrupts the recognition site of the endoprotease, which leads to proteolytic cleavage of the carboxy-terminal 66 amino acids to produce mature ghrelin. The Arg51Gln and Leu72Met SNPs are located in exon 3 and the Gln90Leu SNP in exon 4, respectively. Black boxes = untranslated region; dark gray box = signal peptide; light gray boxes = mature ghrelin peptide; white boxes = cleaved from mature form. (**B**) Standardized pairwise linkage disequilibria (D′, r^2).

Table 1. Allele and genotype frequencies of six ghrelin polymorphisms

Locus	Allele	Allele frequency (*n* = 1014)*	Genotype	Genotype frequency (*n* = 507)
-604G/A	G	0.379	GG	0.134
	A	0.621	GA	0.489
			AA	0.377
-501A/C	A	0.727	AA	0.540
	C	0.273	AC	0.373
			CC	0.087
-473G/A	G	0.988	GG	0.976
	A	0.012	GA	0.024
			AA	—
Arg51Gln	Arg51	0.970	Arg51Arg	0.943
	Gln51	0.030	Arg51Gln	0.055
			Gln51Gln	0.002
Leu72Met	Leu72	0.871	Leu72Leu	0.769
	Met72	0.129	Leu72Met	0.203
			Met72Met	0.028
Gln90Leu	Gln90	0.855	Gln90Gln	0.730
	Leu90	0.145	Gln90Leu	0.250
			Leu90Leu	0.020

* *n,* alleles.

population. The observed allele and genotype frequencies in all genotyped SNPs were in Hardy-Weinberg equilibrium. Location of SNPs in the ghrelin gene and their linkage disequilibrium are shown in Fig. 2. The allele and genotype frequencies for all six studied polymorphisms in the ghrelin gene are shown in Table 1.

When using Haploview software[32] the four most common SNPs (-604G/A, -501A/C, Leu72Met, and Gln90Leu) represented a haplotype block and formed seven major haplotypes (analyzed with solid spine of LD method). The most common haplotype with a frequency of 0.346 included the -604G, -501A, Leu72, and Gln90 alleles of the four SNPs. Subsequently, we compared subjects who had simultaneously the -604GG, -501AA, Leu72Leu, and Gln90Gln genotype (0000), and therefore harbored the most common haplotype, with all other subjects. Further-

Table 2. Baseline characteristics of the DPS subjects

	Genotype		
	-604GG, -501AA, Leu72Leu, and Gln90Gln (*n*)	All other combinations (*n*)	*P*
Sex (M/F)	22/33 (55)	144/308 (452)	.144*
Age (y)	56 ± 7 (55)	55 ± 7 (452)	.370†
Weight (kg)	86.5 ± 13.3 (55)	86.2 ± 14.3 (452)	.925‡
BMI (kg/m^2)	31.0 ± 3.5 (55)	31.3 ± 4.7 (452)	.856†
Waist circumference (cm)	101.9 ± 9.0 (54)	101.1 ± 11.2 (451)	.439†
Waist-to-hip ratio	0.93 ± 0.08 (54)	0.92 ± 0.07 (451)	.353†
Fasting plasma glucose (mmol/L)	6.1 ± 0.7 (55)	6.1 ± 0.8 (452)	.599
2-h plasma glucose (mmol/L)	8.9 ± 1.3 (55)	8.9 ± 1.5 (452)	.882
Fasting serum insulin (pmol/L)	15.0 ± 7.4 (53)	14.7 ± 7.4 (408)	.407
2-h serum insulin (pmol/L)	101.4 ± 103.4 (52)	94.4 ± 58.6 (406)	.563
Total serum cholesterol (mmol/L)	5.55 ± 0.80 (55)	5.62 ± 0.94 (451)	.610
HDL-cholesterol (mmol/L)	1.17 ± 0.26 (55)	1.22 ± 0.30 (451)	.411
LDL-cholesterol (mmol/L)	3.65 ± 0.79 (55)	3.62 ± 0.84 (449)	.809
Triglycerides (mmol/L)	1.62 ± 0.70 (55)	1.74 ± 0.78 (451)	.259‡
Systolic BP (mm Hg)	133 ± 15 (52)	139 ± 18 (450)	.023
Diastolic BP (mm Hg)	84 ± 10 (52)	86 ± 10 (450)	.104
Antihypertensive medication (%)	25.9	37.0	.032†
Hypertension (%)	59.3	64.7	.200†
Alcohol intake (g)	6.4 ± 11.5 (55)	6.8 ± 15.3 (451)	.985‡

DPS = Finnish Diabetes Prevention Study; BP = blood pressure.
Values are means ± SD; ANOVA for comparison among two genotype groups, adjusted for age, gender and BMI as main effects.
* Fisher's exact test; † Mann-Whitney U test; ‡ *P* value adjusted for age and gender.

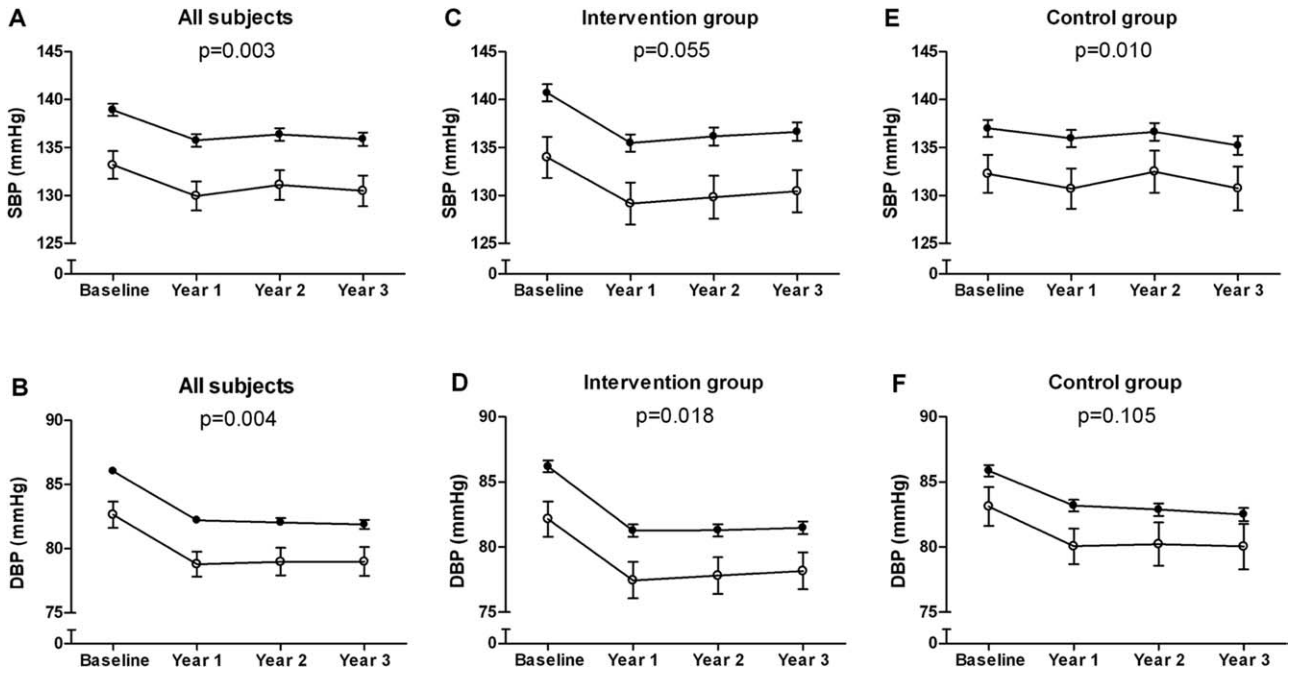


FIG. 3. (**A**) Systolic blood pressure (SBP) levels in the entire study population. Mean values ± SEM calculated from linear mixed model analysis with age, BMI, and alcohol intake as significant covariates included. Open circles = subjects with -604GG, -501AA, Leu72Leu, and Gln90Gln genotypes (n = 54); filled circles = all other genotype combinations (n = 451). (**B**) Diastolic BP (DBP) levels in the entire study population. Mean values ± SEM calculated from linear mixed model analysis with age, gender, antihypertensive medication, BMI, waist circumference, and alcohol intake as significant covariates included. Open circles = subjects with -604GG, -501AA, Leu72Leu, and Gln90Gln genotypes (n = 51); filled circles = all other genotype combinations (n = 448). (**C**) Systolic BP levels in the intervention group. Mean values ± SEM calculated from linear mixed model analysis with age and BMI as significant covariates included. Open circles = subjects with -604GG, -501AA, Leu72Leu, and Gln90Gln genotypes (n = 28); filled circles = all other genotype combinations (n = 231). (**D**) Diastolic BP levels in the intervention group. Mean values ± SEM calculated from linear mixed model analysis with age, gender, and BMI as significant covariates included. Open circles = subjects with -604GG, -501AA, Leu72Leu, and Gln90Gln genotypes (n = 28); filled circles = all other genotype combinations (n = 231). (**E**) Systolic BP levels in the control group. Mean values ± SEM calculated from linear mixed model analysis with age, BMI, and alcohol intake as significant covariates included. Open circles = subjects with -604GG, -501AA, Leu72Leu, and Gln90Gln genotypes (n = 26); filled circles = all other genotype combinations (n = 221). (**F**) Diastolic BP levels in the control group. Mean values ± SEM calculated from linear mixed model analysis with antihypertensive medication, waist circumference, and alcohol intake as significant covariates included. Open circles = subjects with -604GG, -501AA, Leu72Leu, and Gln90Gln genotypes (n = 24); filled circles = all other genotype combinations (n = 219).

more, the -604G/A, -501A/C, and Leu72Met SNPs showed significant associations with BP when analyzed individually (data not shown).

The main clinical and biochemical characteristics of the subjects with the 0000 genotype combination compared to other subjects at baseline are given in Table 2.

Subjects with the 0000 genotype combination had significantly lower systolic (P = .003) and diastolic BP levels (P = .004) than subjects with other genotype combinations at baseline and during 3 years of follow-up (Fig. 3A,B). In the linear mixed models analysis significant covariates for systolic BP were age, BMI, and alcohol intake and for diastolic BP age, gender, antihypertensive medication, BMI, waist circumference, and alcohol intake. When subjects with antihypertensive drug treatment were excluded from the analysis similar trends were obtained (systolic BP, P = .093; diastolic BP, P = .042).

When analyzed separately, subjects with the 0000 genotype combination had lower systolic BP in both the intervention and control group (P = .055 and P = .018, respectively (Fig. 3C,D). Diastolic BP, on the other hand, was lower for subjects with the 0000 genotype combination only in the intervention group (P = .010; Fig. 3E) and not significantly in the control group (P = .105; Fig. 3F).

Although the prevalence of hypertension was not significantly different between subjects with the 0000 genotype combination and the other subjects at baseline (57.7% *v* 64.7%, P = .200), subjects with the 0000 genotype combination had significantly lower prevalence of hypertension at year 1 (40.0% *v* 58.9%, P = .006) and year 2 (41.2% *v* 58.7%, P = .013), and a similar trend was also seen at year 3 (46.8% *v* 59.2%, P = .071). When analyzed with logistic regression these results were reinforced and the 0000 genotype combination turned out to be protective against hypertension in year 1 (OR = 0.408, 95% CI: 0.225–0.739; P = .003), year 2 (OR = 0.438, 95% CI: 0.238–0.804; P = .008), and year 3 (OR = 0.533, 95% CI: 0.285–0.999; P = .050).

Discussion

In this prospective study we show a strong and consistent association between BP levels, prevalence of hypertension, and ghrelin gene polymorphisms. The IGT subjects

who had simultaneously the -604GG, -501AA, Leu72Leu, and Gln90Gln genotypes had the lowest systolic and diastolic BP levels at the baseline and consistently during 3 years of follow-up compared to all other genotypes. These subjects had also a lower prevalence of hypertension and a lower risk of hypertension compared to all other subjects.

Recently, ghrelin has been shown to participate in central cardiovascular and sympathetic regulation.[7–11] Ghrelin may have important direct cardiovascular effects through GH-independent mechanisms.[10,15,16] Ghrelin has been shown to decrease BP,[8,9,13] probably by acting at the nucleus of the solitary tract, one of the most important brain regions regulating BP and the sympathetic nervous system.[10,12,14]

To our knowledge, this is the first study showing a joint effect of several ghrelin polymorphisms on BP levels. Previous studies have investigated the association of single SNPs of the ghrelin gene with hypertension and have generated inconsistent results. We studied the association between a common genotype combination of four ghrelin SNPs and hypertension and BP.

Previously, the Leu72Met SNP has not been associated with hypertension[22] or BP,[24] but Ukkola et al[23] reported that female Met72 carriers had the lowest prevalence of hypertension compared to Leu72Leu subjects. There are no previous data available on the promoter polymorphisms -604G/A, -501A/C, and the novel -473G/A. Here we show for the first time that subjects with a genotype combination that includes the -604GG, -501AA, Leu72Leu, and Gln90Gln genotypes exhibited the lowest systolic and diastolic BP levels compared to the other subjects. In this prospective study, subjects where divided into two groups, an intervention and a control group.[27] When the groups where analyzed separately, diastolic BP was lower for subjects with the 0000 genotype combination only in the intervention group and not in the control group. This could be due to a gene–environment interaction, but on the other hand, the difference between the subjects with the 0000 genotype combination and the other subjects was already apparent at baseline and did not change throughout the study. That this association was not significant could be due to the smaller sample size in the separated data analysis. Systolic BP, however, was lower in subjects with the 0000 genotype combination in both the intervention and control group.

The Gln51 allele of the Arg51Gln SNP has been reported to be a risk allele for hypertension.[21] In our study this SNP was not associated with BP or hypertension.

Low plasma ghrelin levels are inversely correlated with systolic and diastolic BP in different study populations[24,33,34] and associated with hypertension.[24] Data on associations between ghrelin polymorphisms and ghrelin plasma levels are sparse. In previous studies, the Met72Met genotype was associated with the highest ghrelin levels in adults.[23,24] Because we did not measure ghrelin levels in this study, it is difficult to interpret the observed relationship and its biological significance. To our knowledge, no data are available to date concerning the functional relevance of any of the described polymorphisms. Furthermore, none of the described promoter variants is located at binding sites of known transcription factors. Therefore, the mechanisms mediating an association between ghrelin polymorphisms and BP regulation remain yet to be elucidated and other studies are needed to confirm the present findings. However, our results show that ghrelin gene variations were consistently associated with systolic and diastolic BP levels in overweight subjects with impaired glucose tolerance.

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