

Low Plasma Ghrelin Is Associated With Insulin Resistance, Hypertension, and the Prevalence of Type 2 Diabetes

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Experimental studies have suggested that ghrelin plays a role in glucose homeostasis and in the regulation of blood pressure (BP). We therefore assessed the hypothesis that a low ghrelin concentration may be a risk factor for type 2 diabetes and hypertension. We also characterized the effect of the ghrelin Arg51Gln and Leu72Met mutations on ghrelin concentrations in the population-based hypertensive ($n = 519$) and control ($n = 526$) cohorts of our OPERA (Oulu Project Elucidating Risk of Atherosclerosis) study. The fasting plasma ghrelin concentrations of 1,040 subjects were analyzed using the radioimmunoassay method. Insulin sensitivity was assessed using the quantitative insulin sensitivity check index (QUICKI). Ghrelin concentrations were negatively associated with fasting insulin ($P < 0.001$), systolic ($P = 0.026$) and diastolic BP ($P = 0.018$), and the prevalence of type 2 diabetes ($P = 0.015$) and insulin resistance ($P < 0.001$) in the multivariate models. In the control cohort, low ghrelin was associated with hypertension (BP $>140/90$ mmHg) ($P = 0.031$). The subjects with the ghrelin 51Gln allele had lower ghrelin concentrations than the Arg51Arg homozygotes ($P = 0.001$). We conclude that low ghrelin is independently associated with type 2 diabetes, insulin concentration, insulin resistance, and elevated BP. Therefore, it might have some role in the etiology of type 2 diabetes and the regulation of BP. The ghrelin Arg51Gln mutation is associated with low plasma ghrelin concentrations. *Diabetes* 52:2546–2553, 2003

Ghrelin is a recently discovered peptide hormone secreted mainly from the stomach (1). It has a very potent growth hormone (GH)-releasing effect both in animal models and humans (1–3). In addition, it probably has effects independent of GH secretion (4). It is a somatotrophic orexigenic adipogenic hormone that links the regulatory systems for growth and energy balance (5). The effects of ghrelin are mediated

through the GH secretagogue receptor, which is widely distributed in the body (6).

The role of ghrelin in glucose and insulin metabolism has been studied actively. In experimental settings, glucose administration or food intake have been shown to decrease plasma ghrelin concentrations (7–9). Studies of the effects of ghrelin on insulin secretion have shown both stimulatory (10–12) and inhibitory effects (13,14). In human subjects, insulin infusion has been shown to decrease ghrelin concentrations (15,16), whereas parenteral administration of insulin had no effect on ghrelin concentrations (17). On contrary, administration of insulin and leptin induced the increase of ghrelin mRNA in rats (18).

Ghrelin exerts beneficial hemodynamic effects in humans by reducing cardiac afterload and increasing cardiac output (19). The vasodilatory effect is possibly mediated through a GH/IGF-I/nitric oxide-independent mechanism (20).

Based on these recent studies, it appears that ghrelin might have a role in glucose and insulin metabolism and it also may influence blood pressure (BP) levels. Therefore, changes in the activity or concentration of the hormone might constitute a risk factor for impaired glycemic control and elevated BP. It should be noted, however, that the results concerning the role of ghrelin in glucose and insulin metabolism are controversial and reflect the acute effects of ghrelin or the acute changes in the ghrelin concentration. To our knowledge, there are no studies on the long-term effects of ghrelin on insulin and glucose metabolism in the physiological state. Association study is one method to study the potential role of ghrelin in insulin and glucose metabolism.

The aim of this study was to measure the fasting plasma ghrelin concentrations in a large sample of a middle-aged population and analyze these subjects' associations with hypertension and the variables reflecting glucose and insulin metabolism. We hypothesized that low ghrelin constitutes a risk factor for type 2 diabetes and hypertension. A further aim was to characterize the association between ghrelin concentrations and the recently described polymorphisms (Arg51Gln and Leu72Met) of the preproghrelin gene (21).

RESEARCH DESIGN AND METHODS

Subjects. OPERA (Oulu Project Elucidating Risk of Atherosclerosis) is a population-based epidemiological study addressing the risk factors and disease end points of atherosclerotic cardiovascular diseases. The study cohorts and the selection criteria have been described in detail elsewhere (22,23). In

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BP, blood pressure; GH, growth hormone; IGT, impaired glucose tolerance; QUICKI, quantitative insulin sensitivity check index; RIA, radioimmunoassay; WHO, World Health Organization.

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TABLE 1
Percentages of the current users of different cardiovascular medications in the study cohorts

	Hypertensive cohort		Control cohort	
	Men	Women	Men	Women
<i>n</i>	261	258	259	267
β-Blockers	50.6	48.1	7.3	4.1
Diuretics	23.4	38.8	0.8	3.4
Calcium-channel blockers	20.3	22.5	3.5	3.0
ACE inhibitors	40.6	33.7	1.5	2.6
Other BP medication	5.0	7.0	—	—
Any BP medication	91.6	96.1	10.0	10.9
Digitalis	1.5	5.0	1.2	1.5
Lipid-lowering therapy	5.0	2.7	2.3	1.5

short, the hypertensive cohort consists of 600 unrelated subjects (300 men and 300 women aged 40–59 years at the time of recruitment) from the city of Oulu, Finland. The subjects entitled to reimbursement of the costs for antihypertensive medication were randomly selected from the national register. For each hypertensive subject, an age- and sex-matched control subject was randomly selected from the same register, excluding the subjects entitled to reimbursement for antihypertensive medication. The proportions of the current users of different cardiovascular medications in both study cohorts are shown in Table 1. The visits were made in the morning after an overnight fast. Altogether 1,045 subjects participated in the study, which was conducted according to the principles of the Declaration of Helsinki.

Biomedical factors and laboratory analyses. The blood glucose concentration was measured with the glucose dehydrogenase method, and the insulin concentration was measured with the double radioimmunoassay (RIA) method (AIA-Pack IRI; Tosoh, Tokyo). Blood insulin and glucose concentrations were analyzed at 0, 60, and 120 min after administration of 75 g glucose. Routine clinical laboratory tests were carried out in the Central Laboratory of the Oulu University Hospital. Lipid and lipoprotein concentrations were measured as described earlier (24).

Fasting ghrelin concentrations were measured from plasma samples stored at -20°C using a commercial RIA kit (Phoenix Pharmaceuticals, Belmont, CA), which recognizes both acylated and desacylated ghrelin and uses ^{125}I -labeled bioactive ghrelin as a tracer molecule and a polyclonal antibody raised in rabbits against full-length octanoylated human ghrelin. No cross-reactivity was found with human secretin, human vasoactive intestinal peptide, human prolactin-releasing peptide-31, human galanin, human growth hormone releasing hormone (GHRH), neuropeptide Y, orexin A, orexin B, or ghrelin fragments 1–5 and 1–14. No extraction was used in the assay. Since the COOH-terminal amino acid sequence of ghrelin is situated within the amino acid sequence of preproghrelin, the antibody presumably recognizes preproghrelin as well. There are no data available on this cross-reactivity. It is not known whether mutant ghrelin (51Gln) registers on assay or dilutes equally with nonmutant (51Arg) ghrelin. The assay sensitivity was 1.2 pg/tube (ED_{50}), and the inter- and intra-assay coefficient of variations (CVs), as given by the manufacturer, were 7.5 and 4.0%, respectively. The low intra-assay CV (3.8%) has been shown elsewhere also (25). Because of the low intra-assay variation of the method, only a single measurement for each sample was performed. All assays included three control samples from one fresh plasma sample that was transferred to aliquots and frozen at the beginning of the study, used to normalize each test for interassay variability. Based on these results, the mean ghrelin concentration of the control sample was 812 pg/ml and the interassay CV was 11.2%.

Type 2 diabetes and impaired glucose tolerance (IGT) were determined according to World Health Organization (WHO) criteria (26). A person was regarded as diabetic if his or her fasting blood glucose was ≥ 6.1 mmol/l and/or his or her 2-h glucose during an oral glucose tolerance test was ≥ 10.0 mmol/l, or if he or she was using diabetes medication (oral or insulin). A subject had IGT if he or she had a fasting blood glucose < 6.1 mmol/l and his or her 2-h blood glucose during an oral glucose tolerance test was ≥ 6.7 and < 10.0 mmol/l. Insulin sensitivity was assessed using fasting plasma insulin concentrations and a quantitative insulin sensitivity check index $\{\text{QUICKI} = 1/[\log(\text{fasting insulin}) + \log(\text{fasting glucose})]\}$ (27). The subjects with insulin sensitivity below the lowest quartile of the control cohort ($\text{QUICKI} < 0.563$) were regarded as insulin resistant, as suggested by the WHO expert committee (26). Hypertension was determined according to the current WHO guidelines (BP $> 140/90$).

The questionnaire presented to all participants elicited detailed information about their smoking habits, alcohol consumption, physical activity, use of medication, and past medical history. Alcohol consumption was calculated as grams of absolute alcohol consumed per week and smoking as the number of cigarettes smoked per day. Physical activity was determined by a separate item and scored into five categories (28).

DNA analyses. Genomic DNA was extracted from blood leukocytes. The 618-bp DNA fragment covering exons 1 and 2, which encompass the entire ghrelin product, was amplified using the PCR technique and the following primers: forward primer, 5'-GCTGGGCTCCTACCTGAGC-3'; reverse primer, 5'-GGACCCTGTTCCTACTGCCAC-3' (21). The Arg51Gln mutation was identified using the restriction endonuclease *SacI*, which retains the mutated site (guanine replaced by adenine) at base 346 in exon 2 of the preproghrelin gene undigested. The preproghrelin Leu72Met polymorphism is caused by a cytosine-to-adenine transition at base 408 in exon 2 of the preproghrelin gene, which leads to abolishment of the *BsrI* restriction site. The amplified products were digested at 37°C (*SacI*) or 65°C (*BsrI*) overnight with 5 units of the enzyme. The fragments were separated on a 1.5% agarose gel and visualized under ultraviolet light after staining with the GelStar nucleic acid gel stain (BioWhittaker Molecular Applications, Rockland, ME).

Statistical methods. To compare the means of the variables measured, Student's *t* test, ANOVA, and ANCOVA were used. The association between ghrelin and the variables studied was assessed using linear and logistic regression analyses. The following variables were entered into the multivariate models: sex, study group, BMI, age (analyses of glucose and insulin metabolism) or sex, study group, BMI, age, alcohol consumption, and physical activity (hypertension and BP levels). Because the ghrelin concentrations were analyzed in a nonrandom order (hypertensive men were analyzed at first, followed by hypertensive women, control men, and control women), we wanted to control for the potential effect of the interassay variation causing systematic error on the ghrelin concentrations. Therefore, we used linear regression analysis to adjust the individual ghrelin concentrations for the interassay variation. Adjusted ghrelin levels were used in the analyses. A χ^2 test was performed to assess whether the observed genotype frequencies were in the Hardy-Weinberg equilibrium.

The study cohorts and sexes were analyzed separately if there was a significant interaction between the study group or sex and the variable studied. The three subjects found to have type 1 diabetes were excluded from this study. Three subjects with very high ghrelin concentrations were excluded from the analyses as outliers. Altogether, the fasting plasma ghrelin concentrations of 1,034 subjects were included in the analyses.

Log-transformed (triglycerides, HDL cholesterol, glucose, and insulin) values were used as appropriate to normalize the skewed distributions. All calculations were made with the SPSS statistical package (version 9.0; SPSS Inc.). A *P* value < 0.05 was regarded as significant. Homogeneity of variances was tested using Levene's test. Post hoc tests were performed using Tukey's method. Bonferroni correction was used in multiple comparisons. All the tests performed were two-sided.

RESULTS

The mean fasting plasma ghrelin concentration of the whole study cohort was 668 pg/ml (range 117–1,513). The mean values of ghrelin concentration were 657 and 678 pg/ml in men and women, respectively ($P = 0.169$), and 661 and 674 pg/ml in the hypertensive and control cohorts, respectively ($P = 0.359$). Table 2 shows the characteristics of the study cohort according to ghrelin quartiles. Low ghrelin was associated with high BMI, high waist circumference, high systolic and diastolic BP, and high fasting blood glucose and plasma insulin, leptin, and triglyceride concentrations in the analyses adjusted for sex and study group. When BMI was added to the covariates, only the associations between ghrelin and high diastolic BP and high insulin concentrations remained statistically significant (Table 2). There was a positive association between ghrelin and HDL cholesterol, but only in the control cohort (Table 2). The interaction term between age and sex was significant as a determinant of ghrelin concentrations ($P < 0.05$). Therefore, the sexes were analyzed separately. In the multivariate analysis adjusted for study group and BMI, age was positively associated with ghrelin concen-

TABLE 2
Characteristics of the study subjects according to the ghrelin quartiles

	First quartile	Second quartile	Third quartile	Fourth quartile	P*	P†
n (M/F)	258 (141/117)	259 (125/134)	259 (122/137)	258 (128/130)		
Ghrelin (pg/ml)	367 (358-376)	573 (566-579)	744 (737-750)	987 (970-1004)		
Hypertensive cohort (%)	55.4	43.2	49.0	51.6	0.364‡	
Age (years)						
Men	52 (51-53)	49 (48-50)	50 (49-51)	51 (50-52)	0.097	
Women	51 (50-53)	51 (50-52)	52 (51-53)	53 (52-54)	0.107	
Leptin (ng/ml)	11.4 (10.6-12.2)	10.8 (10.0-11.7)	10.2 (9.4-11.0)	9.7 (8.9-10.5)	0.001	0.288
BMI (kg/m ²)	28.5 (28.0-29.1)	27.8 (27.3-28.3)	27.4 (26.9-28.0)	27.0 (26.5-27.5)	<0.001	
Waist (cm)	92 (91-94)	91 (90-92)	90 (89-91)	89 (88-90)	<0.001	
Height (cm)	168 (167-169)	168 (167-169)	168 (167-169)	168 (168-169)	0.281	
Systolic BP (mmHg)	151 (148-153)	149 (146-152)	148 (145-150)	146 (144-149)	0.007	0.059
Diastolic BP (mmHg)	90 (89-91)	90 (88-91)	89 (88-90)	88 (87-89)	0.006	0.037
HbA _{1c} (%)	6.6 (6.5-6.8)	6.7 (6.6-6.9)	6.7 (6.5-6.9)	6.4 (6.3-6.6)	0.098	0.197
Fasting glucose (mmol/l)	7.4 (7.0-7.7)	7.2 (6.8-7.5)	7.0 (6.6-7.4)	6.8 (6.4-7.2)	0.013	0.160
AUC glucose (mmol · l ⁻¹ · h ⁻¹)	13.4 (12.8-14.1)	13.2 (12.5-13.8)	12.9 (12.3-13.6)	12.4 (11.8-13.1)	0.024	0.312
Fasting insulin (mU/l)	15.5 (14.2-16.8)	14.2 (12.9-15.5)	13.1 (11.8-14.4)	11.7 (10.4-13.0)	<0.001	<0.001
AUC insulin (mu · l ⁻¹ · h ⁻¹)	162 (148-177)	145 (131-159)	137 (123-151)	124 (110-138)	<0.001	0.008
QUICKI	0.59 (0.58-0.60)	0.60 (0.59-0.61)	0.60 (0.59-0.61)	0.64 (0.62-0.65)	<0.001	<0.001
Type 2 diabetes (%)	13.6	8.1	7.3	6.2	0.002‡	0.011‡
Total cholesterol (mmol/l)	5.7 (5.5-5.8)	5.7 (5.6-5.8)	5.8 (5.7-5.9)	5.7 (5.5-5.8)	0.974	
Triglycerides (mmol/l)	1.7 (1.5-1.8)	1.6 (1.4-1.7)	1.6 (1.5-1.7)	1.5 (1.4-1.6)	0.037	0.407
HDL cholesterol (mmol/l)						
Hypertensive cohort	1.3 (1.2-1.3)	1.3 (1.2-1.4)	1.3 (1.3-1.4)	1.3 (1.3-1.4)	0.315	
Control cohort	1.3 (1.2-1.4)	1.3 (1.3-1.4)	1.4 (1.3-1.4)	1.5 (1.5-1.6)	<0.001	<0.001

Data are % or means (95% CI). Means are adjusted for sex and study group. P values obtained from linear or logistic (‡) regression analyses adjusted for sex and study group (*) or sex, study group, and BMI (†). There was no significant difference in sex ratio or LDL cholesterol between the quartiles. AUC, area under the curve.

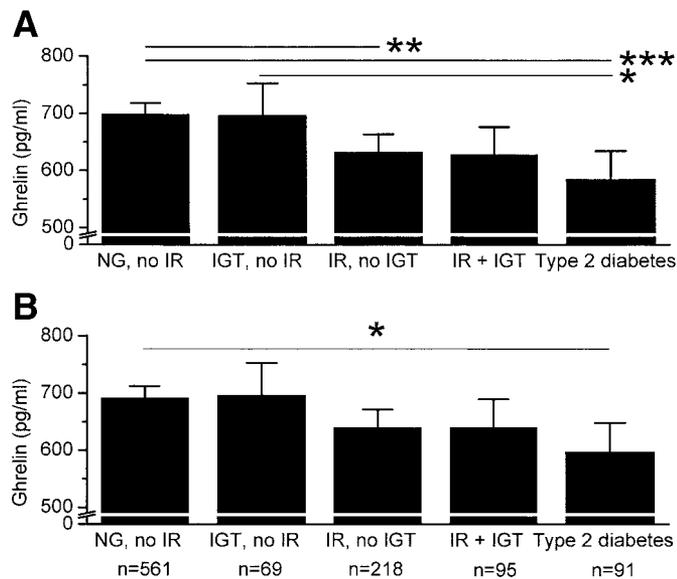


FIG. 1. Fasting plasma ghrelin concentrations in normoglycemic (NG) subjects with no insulin resistance (IR) and in subjects with IGT but no IR, in subjects with IR and no IGT, in subjects with IR and IGT, and in subjects with type 2 diabetes. Data are means and 95% CIs. **A:** Ghrelin concentrations without adjustment for BMI. ANCOVA: $P < 0.001$, $***P < 0.001$, $**P = 0.007$, $*P = 0.045$ (significance for the difference between the groups). **B:** Ghrelin concentrations adjusted for BMI. ANCOVA: $P = 0.007$, $*P = 0.014$.

trations in women ($P = 0.038$), but no similar association was seen in men ($P = 0.128$).

Ghrelin and type 2 diabetes. There was a significant difference in the prevalence of type 2 diabetes between the ghrelin quartiles, so that the highest prevalence was among the subjects with the lowest ghrelin concentration (Table 2). Figure 1 shows the ghrelin concentrations in

relation to glycemic status with and without adjustment for BMI. The ghrelin concentrations of type 2 diabetic subjects were low compared with those of the nondiabetic subjects (586 vs. 675 pg/ml; $P = 0.001$, $P = 0.012$ after adjustment for BMI). The association of low ghrelin with the prevalence of type 2 diabetes ($P = 0.015$) and insulin resistance ($P < 0.001$) persisted in the multivariate models, but the association between ghrelin and IGT was not statistically significant ($P = 0.602$). In the multivariate models, the inverse relationship between ghrelin and fasting insulin ($P < 0.001$) and area under the curve insulin ($P = 0.003$) as well as the positive relationship between the QUICKI index and ghrelin ($P < 0.001$) remained.

Ghrelin, BP, and hypertension. Figure 2 shows the variation of systolic and diastolic BP relative to the ghrelin quartiles in the study cohorts with and without adjustment for BMI. Because the interaction term between the study cohort and ghrelin as a predictor of BP levels was insignificant (>0.05), the study cohorts were pooled together. Low ghrelin was a significant predictive factor for both systolic ($P = 0.026$) and diastolic ($P = 0.018$) BP in the multivariate models of the pooled data. Because of the marked difference in the BP levels between the study cohorts, the cohorts were also analyzed separately. In that case, the association was evident with systolic ($P = 0.047$) and diastolic ($P = 0.007$) BP in the hypertensive cohort. No significant association was detected in the control cohort ($P = 0.279$ and $P = 0.526$ for systolic and diastolic BP, respectively). However, low ghrelin was associated with the prevalence of hypertension (BP $>140/90$ mmHg) in the control cohort in the logistic regression analysis ($P = 0.031$), but the effect did not remain statistically significant in the multivariate model ($P = 0.101$).

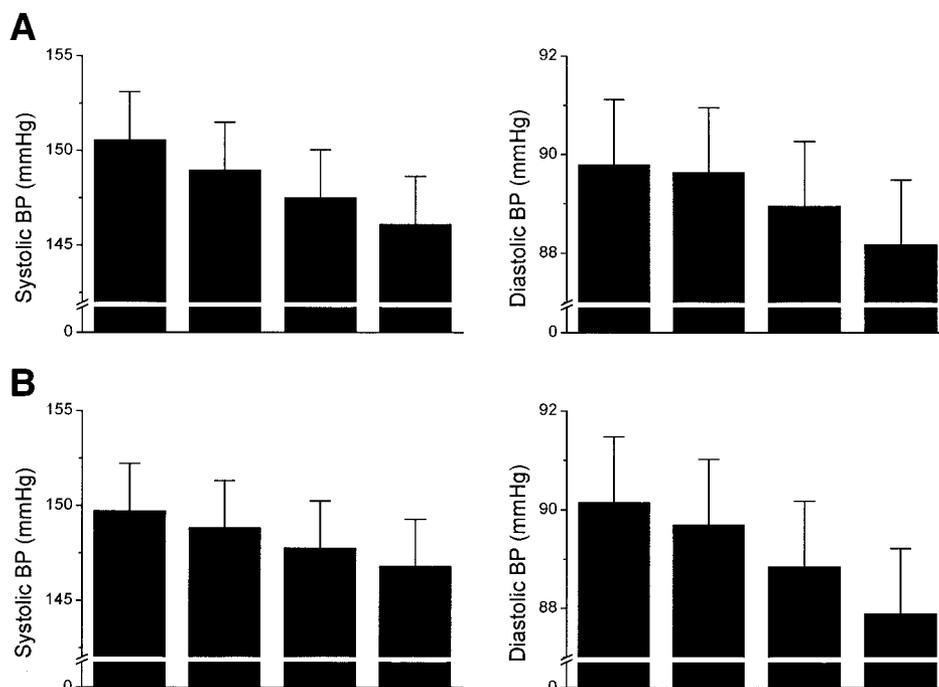


FIG. 2. Variation of systolic and diastolic BP in the pooled data according to the ghrelin quartiles. Data are means and 95% CIs. The first bar represents the first ghrelin quartile, the second represents the second ghrelin quartile, etc. **A:** Means adjusted for sex and study cohort; $P = 0.007$ for systolic BP and $P = 0.006$ for diastolic BP in the linear regression analysis adjusted for sex and study cohort. **B:** Means adjusted for sex, study cohort, and BMI; $P = 0.059$ for systolic BP and $P = 0.037$ for diastolic BP in the linear regression analysis adjusted for sex, study cohort, and BMI.

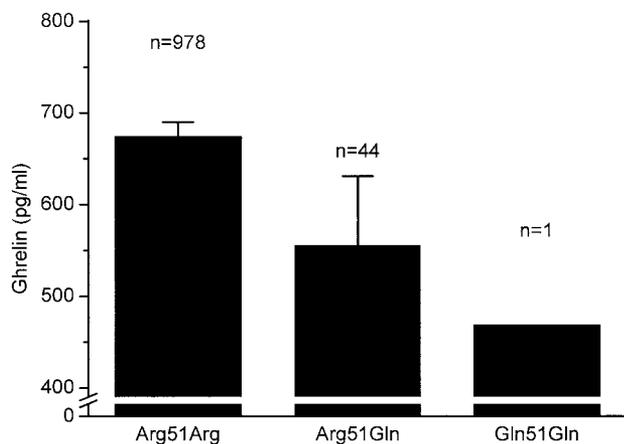


FIG. 3. Fasting plasma ghrelin concentrations according to the Agr51Gln genotype. Data are means and 95% CIs. $P < 0.001$ for the difference between the subjects homozygous for Arg51 and the 51Gln carriers after adjustment for BMI, sex, and study group.

Ghrelin mutations and ghrelin concentration. The fasting plasma ghrelin concentrations of the Arg51Gln and Leu72Met genotypes are presented in Figs. 3 and 4, respectively. The mean value of ghrelin was lower in the subjects with the 51Gln allele compared with the Arg51Arg homozygotes (545 vs. 675 pg/ml, $P < 0.001$). The effect of the Arg51Gln mutation remained in the linear regression analysis adjusted for BMI, sex, and study group ($P < 0.001$).

In the analysis of the Leu72Met polymorphism, the interaction term between the study group and the polymorphism was significant ($P < 0.05$), and, consequently, the study groups were analyzed separately. There was no difference in ghrelin concentrations between the Leu72Met genotypes in the control cohort (ANOVA, $P = 0.773$), whereas in the hypertensive cohort, the polymorphism was a significant predictor of ghrelin level (ANOVA, $P = 0.006$) (Fig. 4). The hypertensive subjects with the Leu72Met genotype had lower ghrelin concentrations than the subjects with the Leu72Leu genotype ($P = 0.022$). The effect was also evident after adjusting for BMI and sex (ANCOVA, $P = 0.006$, $P = 0.020$, for the difference between the Leu72Met and Leu72Leu genotypes). When Met72 carrier status was considered, there was no statis-

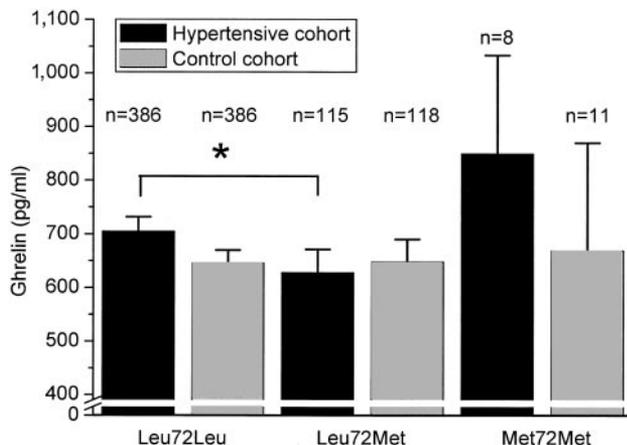


FIG. 4. Fasting plasma ghrelin concentrations in the hypertensive and control cohort according to the Leu72Met genotypes. Data are means and 95% CIs. * $P = 0.020$ for the difference between the groups after adjustment for BMI and sex.

tically significant difference in the mean ghrelin values of the Met72 carriers and the subjects homozygous for the Leu72 allele in the hypertensive cohort (660 vs. 809 pg/ml, $P = 0.094$). There were no differences in the mean values of BP, BMI, waist circumference, plasma lipid, insulin, leptin, or blood glucose between the subjects homozygous for the Leu72 allele and the Met72 carriers in the analyses adjusted for sex and study group (data not shown).

DISCUSSION

Our data characterize the variation of fasting plasma ghrelin concentrations in a randomly selected sample of a middle-aged hypertensive control population. To our knowledge, this is the first study exploring the ghrelin concentrations in a randomly selected sample of subjects at the population level. Our study suggests that low ghrelin is independently associated with elevated BP level and insulin concentration and the prevalence of type 2 diabetes and insulin resistance.

We found ghrelin concentrations of subjects with type 2 diabetes to be lower than those of subjects without type 2 diabetes. In line with the previous studies (7,29), our study also demonstrated adiposity to be an important determinant of ghrelin concentrations. Therefore, the lower ghrelin concentration in type 2 diabetic subjects could be due to their higher adiposity. However, the difference remained after adjustment for BMI, and the association between low ghrelin and the prevalence of type 2 diabetes persisted independently of sex, study group, BMI, and age in the multivariate model. Furthermore, the inverse association between ghrelin and fasting insulin concentrations as well as the association between ghrelin and insulin sensitivity (QUICKI) remained after the adjustments. Thus, our results suggest that low ghrelin is independently associated with fasting insulin concentrations, insulin resistance, and type 2 diabetes. Indeed, fasting ghrelin concentrations have been shown to be reduced in healthy offspring of type 2 diabetic subjects (30). These findings support the view that low ghrelin could have a causative role in the development of type 2 diabetes. Low ghrelin concentrations have been associated previously with insulin resistance in subjects with polycystic ovary syndrome (31,32) and in obese children and adolescents (33). The negative correlation between fasting ghrelin and insulin has also been reported earlier (29). Our findings are in accordance with these results, showing that ghrelin level is negatively associated with insulin concentrations and the prevalence of type 2 diabetes and positively associated with insulin sensitivity.

Based on current knowledge on the interplay between plasma insulin, glucose, and ghrelin, it is difficult to explain the mechanism behind the association between low ghrelin concentrations and the increased prevalence of type 2 diabetes. Studies about the effects of ghrelin on insulin secretion have shown both stimulatory (10–12) and inhibitory effects (13,14). Two studies have reported a decrease of plasma ghrelin caused by insulin infusion both in hypoglycemic (16) and euglycemic (15,16) conditions. These findings suggest that ghrelin concentrations might be downregulated in the hyperinsulinemic state and that ghrelin per se may not necessarily play any role in the development of insulin resistance and type 2 diabetes.

However, nihilistic (17) and even contrary results also exist (18). A recent experiment demonstrated that only supraphysiological concentrations of insulin decrease ghrelin concentrations, whereas somatostatin causes a marked decrease in ghrelin concentrations without changes in insulin levels (34). *In vitro* ghrelin modulates insulin signaling in hepatoma cells, implying peripheral actions of ghrelin in glucose homeostasis as well (35).

The contradictory results concerning the role of ghrelin in glucose and insulin metabolism might be due to the different experimental settings of the studies and represent only the acute effects of the hormone or acute changes in its concentration. It is important to point out that the studies on the long-term physiological effects of ghrelin on insulin and glucose metabolism remain unknown. However, the present study suggests that ghrelin might play a role in insulin and glucose metabolism.

Theoretically, low ghrelin could affect the development of type 2 diabetes and insulin resistance in several ways. One could speculate that ghrelin deficiency in itself and/or the decreased somatotrophic effects associated with ghrelin deficiency decrease insulin sensitivity and eventually lead to type 2 diabetes. Decreased ghrelin expression has been suggested to be associated with age and hyposomatotropism in the elderly (36,37). Therefore, an age-dependent decrease in ghrelin concentrations could be associated with decreased insulin sensitivity. In our study cohort consisting of subjects aged 40–62 years, no such effect was evident. On contrary, we found a positive association between ghrelin concentrations and age in women. In addition, the inverse association between ghrelin concentrations and the prevalence of type 2 diabetes was independent of age.

Our study demonstrates for the first time the negative correlation between both systolic and diastolic BP and fasting plasma ghrelin concentrations in a population-based cohort. The high BMI levels of the subjects with low ghrelin partly explained the association between low ghrelin concentrations and elevated BP. The multivariate analysis of the pooled data, however, showed the effect of ghrelin to be independent of BMI and the other most commonly recognized risk factors for hypertension. When the study cohorts were analyzed separately, the association remained only in the hypertensive cohort, implicating that the effect of low ghrelin on BP levels is stronger in a hypertensive state and only becomes evident in a large data set. In the control cohort, the negative association between the prevalence of hypertension and the ghrelin concentrations was diluted after adjustment for BMI. The association between BP and ghrelin has been reported earlier in pregnant women (38), and in experimental settings, ghrelin exerts beneficial hemodynamic effects by decreasing the mean arterial pressure and increasing the cardiac output (19,39). The vasodilatory effects of ghrelin *in vitro* suggest that the mechanism is independent of the GH/IGF-1/nitric oxide axis (20). These findings suggest that ghrelin might have a role in the regulation of BP, especially in the hypertensive state.

The amino acid Arg51 of the ghrelin gene is a target site for endoprotease action, which leads to proteolytic cleavage of the COOH-terminal 66 amino acids to produce mature ghrelin. The Arg51Gln mutation disrupts the rec-

ognition site in the last codon of the mature ghrelin product (21). Ukkola et al. (40) have shown previously that subjects with the Arg51Gln mutation have low ghrelin concentrations compared with the Arg51Arg subjects. This finding is supported by our results, which show the subjects with the 51Gln allele to have low ghrelin concentrations. The mutation is quite rare. However, it appears to have obvious phenotypic consequences: the subjects with the 51Gln allele have low ghrelin concentrations and are at higher risk to have type 2 diabetes and hypertension (41).

The mutation at codon 72 of the preproghrelin gene (Leu72Met) might have effects on the products of preproghrelin, which could, theoretically, have some as yet unknown functional significance, as suggested by Ukkola et al. (40). They reported the ghrelin 72Met allele to be protective against fat accumulation and associated metabolic comorbidities without any effect on ghrelin concentrations (40). Our data do not support this hypothesis because the Leu72Met polymorphism was not associated with the metabolic variables studied and the subjects with the Met72 allele had low ghrelin concentrations in the hypertensive cohort, which should be associated with adverse rather than protective metabolic effects.

To obtain a measure of overall ghrelin concentrations, we used fasting plasma ghrelin concentrations, which have been previously shown to correlate strongly with the 24-h integrated area under the curve values (42). The polyclonal antibody used recognizes the COOH-terminal amino residue sequence of ghrelin, which is identical in preproghrelin. Therefore, the ghrelin concentrations of this study reflect both ghrelin and preproghrelin immunoreactivity. Because the Arg51Gln mutation changes the last COOH-terminal amino acid of ghrelin, it is presumable that the RIA equally measures both normal and mutated ghrelin. However, there are no data available on the effect of this amino acid change on the binding affinity of the antibody to the mutated ghrelin molecule. Therefore, the association of the Arg51Gln mutation and ghrelin concentration should be verified, e.g., by using a monoclonal ghrelin antibody with no binding affinity to the last amino acid of the molecule.

There are very few studies on the stability of ghrelin or the effect of different storage conditions on the stability of the hormone. It has been shown that repeated freezing and thawing has no effect on the ghrelin concentration and that ghrelin was stable up to 3 days when stored at 4°C (25). In our analyses, the mean concentration of the control sample and 10 other fresh plasma samples were of the same magnitude as measured in the study samples. Thus, we have only indirect evidence that ghrelin appears to be stable at –20°C over time. Given that some degradation of the molecule occurred in these storage conditions, degradation probably would have happened equally in all samples and would have no major effect on the conclusions of the study.

In conclusion, we have shown in our population-based study that low ghrelin is independently associated with both insulin concentrations and the prevalence of type 2 diabetes and insulin resistance. This finding suggests that ghrelin might have some role in the etiology of type 2 diabetes. We also demonstrated the negative association between ghrelin and BP, which implicates that ghrelin

could participate in the regulation of BP, especially in the hypertensive state. Furthermore, the present study supports the hypothesis that the ghrelin Arg51Gln mutation is associated with ghrelin concentrations. Prospective studies are needed to elucidate the potential causal role of ghrelin in the development of type 2 diabetes and hypertension.

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