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# Fasting plasma total ghrelin concentrations in monozygotic twins discordant for obesity

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**Abstract**

Ghrelin is a hormone that is involved in the regulation of food intake. Neuronal, endocrine, and genetic factors have been shown to regulate plasma ghrelin levels; but the determinants of fasting ghrelin concentrations are not yet fully understood. The main aim was to explore the roles of adiposity and genetic differences in determining fasting plasma total ghrelin levels. We measured total ghrelin levels in a population of 23 monozygotic twin pairs discordant for obesity. In addition, 2 variants of ghrelin gene, namely, Arg51Gln and Leu72Met, were genotyped in 3 populations of monozygotic twin pairs: 23 obesity-discordant, 43 lean-concordant, and 46 obesity-concordant twin pairs. In discordant twins, lean co-twins had higher fasting plasma total ghrelin levels (950 pg/mL, SD = 328 pg/mL) than obese twins (720 pg/mL, SD = 143 pg/mL; $P = .003$). Arg51Gln-polymorphism of the ghrelin gene was equally distributed between the twin groups. However, there were significant differences in genotype frequencies at the Leu72Met polymorphism between the discordant and obese-concordant groups ($P = .003$) and between the discordant and lean-concordant groups ($P = .011$), but not between the 2 concordant groups. In the discordant group, there were fewer Met carriers (4%) than among the obese (17%) or the lean-concordant groups (15%). Plasma total ghrelin levels are affected by acquired obesity independent of genetic background. The Leu72 allele is particularly common among monozygotic twins discordant for obesity, suggesting that this ghrelin allele is more permissive in the regulation of energy balance. The ghrelin gene may thus play a role in the regulation of variability of body weight, such that Leu72 allele carriers are more prone to weight variability in response to environmental factors.


## 1. Introduction

Ghrelin is a peptide hormone of 28 amino acids with a potent growth hormone–releasing effect [1]. In addition, administrated ghrelin has been shown to stimulate feeding independently of growth hormone secretion and cause weight gain in rodents [2]. In humans, exogenous ghrelin increases appetite and food intake [3]. In physiologic conditions, plasma levels of ghrelin increase before and decrease after food intake; and based on these findings, ghrelin is thought to be a meal-initiating factor [4]. Fasting ghrelin levels are lower in obese than in normal-weight subjects [5]. Low fasting ghrelin concentrations have been associated with insulin resistance, hypertension, and type 2 diabetes mellitus [6]. It has been suggested that plasma ghrelin concentrations might be down-regulated in human obesity because of the chronic positive energy balance and the high insulin and leptin concentrations [5]. However, the regulation of ghrelin concentrations is not known in detail.

An overfeeding twin study suggested that genetic factors might play a role in the determination of basal plasma levels of total ghrelin [7]. Some genetic variations in the ghrelin gene have been identified, and some have been shown to associate with ghrelin levels and with obesity. For example

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the Arg51Gln mutation, which changes the carboxy terminal amino acid of the mature ghrelin, is associated with low ghrelin concentrations [3]. Another polymorphism, a nucleotide 408C-A transversion, which causes a Leu72Met amino acid change, has been shown to associate with weight-related phenotypes in several studies [8-11]. This polymorphism is located outside the mature ghrelin transcript.

Genetic factors play an important role in the responsiveness to changing environmental conditions. Some 20 years ago, Kåre Berg [12-14] put forward the variability gene concept and provided empirical evidence in its favor based on studies of intrapair differences of monozygotic (MZ) pairs differing in a particular phenotype. This can also be considered a form of gene-environmental interaction, such that certain genotypes are more sensitive to environmental influences than others; that is, certain MZ pairs exhibit greater intrapair variability than others. A gene-environment interaction effect was also evident in the Quebec long-term overfeeding study with MZ twins [15]. Another unique way to study gene-environmental interaction is to study MZ twin pairs discordant for a relevant phenotype, such as obesity. Intrapair differences between the obese and lean members of a given MZ twin pair are postulated to be caused by nongenetic factors such as epigenetic events [16-18] or prenatal or postnatal environmental factors [19]. The magnitude of intrapair differences in phenotypes in a set of discordant MZ twin pairs is modulated by the combined effects of genes, environmental factors, and their interactions. For example, it has been shown that twin pairs characterized by both the apolipoprotein E4 phenotype and a high abdominal visceral fat (AVF) level exhibit particularly high intrapair differences in serum triglycerides and their subfractions [20].

Rönnemaa et al [21] showed that leptin concentrations in obesity-discordant twin pairs were higher in female than male twins and higher in obese subjects compared with lean twin brothers or sisters. Among younger MZ twin pairs discordant for adult-onset obesity, acquired obesity is associated with increases in liver fat, vascular abnormalities, insulin resistance, and changes in adipose tissue metabolism [22-27].

The aim of the present study was to explore whether there are differences in fasting plasma total ghrelin concentrations between obese and lean members of adult MZ twin pairs discordant for obesity. In addition, the genotype distributions of 2 ghrelin polymorphisms, namely, Agr51Gln and Leu72Met, were investigated in MZ twin pairs discordant for obesity, obese-concordant MZ twin pairs, and lean-concordant MZ twin pairs.

## 2. Subjects and methods

The present study is based on a sample of 23 MZ twin pairs (9 male pairs and 14 female pairs) discordant for obesity. There was, on average, an 18-kg intrapair difference in body weight between the normal-weight and the overweight member across all pairs. The mean intrapair difference in body mass index (BMI) was 5.2 kg/m$^2$ (SD = 1.5 kg/m$^2$) in male and 7.6 kg/m$^2$ (SD = 3.1 kg/m$^2$) in female twins. The study subjects had no diseases and were not receiving continuous treatment with medication. Discordant twin pairs were selected from the Finnish Twin Cohort as described earlier [20].

The methods concerning phenotype measurements of the twins discordant for obesity have been described previously in detail [20]. Adiposity was expressed as the percentage of body fat. The latter was determined using a 4-component model. The density of the whole body was estimated by underwater weighing, corrected for information on body water and mineral mass. The proportion of fat tissue was calculated from the density of the whole body according to the formula of Siri [28]. The distribution of body fat was measured by magnetic resonance imaging. Imaging was done at 0.1 T (Mega4; Instrumentarium, Helsinki, Finland). Axial and sagittal localizers were used to obtain a transaxial T1-weighted image (relaxation time/echo time, 155/20; slice thickness, 10 mm) at the level of the fourth lumbar vertebra. Abdominal visceral and subcutaneous (ASF) fat areas were measured. Magnetic resonance imaging was not available for 3 pairs of twins (2 female pairs, 1 male pair) either because the participants had claustrophobia or because the imaging equipment was temporarily malfunctioning [29].

Blood pressure (BP) was measured with a standard mercury sphygmomanometer in sitting position after a 5-minute rest. The latter of 2 measurements was used in analyses. The fifth phase of the Korotkoff sounds was taken as diastolic BP. Venous blood samples were drawn after a 12-hour fast. Serum cholesterol concentration was determined using an enzymatic method [30]. After removing very low-density lipoprotein, low-density lipoprotein (LDL) was precipitated from the infranatant (high-density lipoprotein [HDL] + LDL) with dextran sulfate 500 000–magnesium chloride, according to the method of Kostner [31].

Glucose metabolism was assessed in a 2-hour glucose (75 g) tolerance test with glucose and insulin measurements at 0, 30, 60, 90, and 120 minutes. Serum glucose was measured by the glucose dehydrogenase method (Merck Diagnostica, Darmstadt, Germany). Plasma insulin was measured by radioimmunoassay (RIA) (Pharmacia Diagnostics, Uppsala, Sweden). Area under the plasma insulin curve (AUC) was used as an estimate of insulin resistance. Fasting plasma leptin levels were determined in duplicate by an RIA kit (Linco Research, St Charles, MO) as previously described [32]. Fasting plasma total ghrelin concentrations were assessed by RIA using a commercial kit (Peptide Radioimmunoassay Kit; Phoenix Pharmaceuticals, Belmont, CA). This kit measures the amount of total ghrelin. The antibodies of the kit recognize both the acylated and the desacylated 28 amino acid–containing ghrelin molecules in the plasma. The inter- and intraassay coefficients of variation (CVs), as given by the manufacturer, were 7.5% and 4.0%,

respectively. Intraassay CV in our analyses varied between 0.23% and 7.8%; mean CV was 2.12%.

The control population consists of 43 age- and sex-matched MZ twin pairs, also from the Finnish Twin Cohort, concordant for normal BMI (<25 kg/m$^2$) and 46 MZ twin pairs concordant for obesity (BMI >30 kg/m$^2$). In addition to sex, age, and BMI data, DNA was available for all these twin pairs. Two variants of ghrelin, namely, Arg51Gln and Leu72Met, were genotyped in obesity-discordant and control MZ twins by restriction fragment length polymorphism technique, as described earlier [9]. The subjects had provided informed consent to participate in studies of the genetics of obesity and related disorders. Ethics committees of the Turku University Hospital and Helsinki University Hospital approved the data collection protocols.

### 2.1. Statistical analysis

Intrapair differences in ghrelin levels and other variables were tested using paired-sample *t* tests. Differences between sexes were compared using Δ values (difference between obese and lean co-twin) with independent-samples *t* tests. The Δ values were also used when correlations of intrapair differences in ghrelin levels and those of other variables were computed. Pearson correlations were adjusted for sex using linear regressions. Concentrations of ghrelin, fasting insulin, fasting glucose, triglycerides, and HDL cholesterol and values of AVF, AUC-insulin, AUC-glucose, and systolic and diastolic BP showed skewed distribution and were normalized using a logarithmic transformation before the analyses. Differences in genotype frequencies between groups were tested using Fisher exact test. Analyses were performed with the SPSS (versions 14.0 and 15.0; SPSS, Chicago, IL), and Stata 8.2 (Stata, College Station, TX). Statistical significance was set at *P* less than .05.

## 3. Results

### 3.1. Ghrelin concentrations in MZ twins discordant for obesity

There were significant intrapair differences between obese and lean co-twins in BMI, waist-hip ratio (WHR), AVF and ASF, total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides in both male and female MZ obesity-discordant twin pairs. In addition, in male twin pairs, there were intrapair differences in leptin and fasting plasma glucose concentrations, whereas in female twin pairs, there were intrapair differences in percentage of body fat and diastolic BP as shown in Table 1 and as earlier described [20,21,33–35]. Compared with men, female subjects were significantly more discordant for BMI (*P* = .043) and for ASF accumulation (*P* = .002).

There was also a significant intrapair difference between obese and lean co-twins in fasting plasma total ghrelin concentrations (*P* = .003). The mean difference was 230 pg/mL (SD = 327 pg/mL). The lean co-twins had higher ghrelin levels (mean, 950 pg/mL; SD = 328 pg/mL) than did their obese co-twins (mean, 720 pg/mL; SD = 143 pg/mL). When examined by sex, the intrapair differences in total ghrelin concentrations were significant in female pairs (*P* < .001) but not in male pairs (*P* = .415) (Table 1 and Fig. 1). In female pairs, the mean intrapair difference of fasting total ghrelin was 301 pg/mL (SD = 242 pg/mL); and that of men was 120 pg/mL (SD = 419 pg/mL). In female twins, the mean concentrations of ghrelin were 726 pg/mL (SD = 113 pg/mL) in obese twins and 1027 pg/mL (SD = 275 pg/mL) in lean co-twins. In male twins, the mean ghrelin concentration of the obese twins was 710 pg/mL (SD = 166 pg/mL); and that of lean twins was 831 pg/mL (SD = 383 pg/mL) (Table 1).

Table 1
Clinical characteristics of MZ twin pairs discordant for obesity

| | Male (9 pairs) | | | Female (14 pairs) | | |
|---|---|---|---|---|---|---|
| | Obese | Lean | *P* | Obese | Lean | *P* |
| Age (y) | 44 (7) | 44 (7) | | 47 (7) | 47 (7) | |
| Weight (kg) | 88.9 (8.7) | 72.9 (9.5) | <.001 | 79.1 (6.4) | 59.8 (6.0) | <.001 |
| BMI (kg/m$^2$) | 28.8 (1.6) | 23.6 (0.9) | <.001 | 30.0 (3.2) | 22.4 (1.5) | <.001 |
| Percentage of body fat (%) | 25.7 (3.0) | 21.8 (2.7) | NS | 38.5 (3.9) | 26.7 (5.3) | <.001 |
| WHR | 0.97 (0.07) | 0.90 (0.05) | .004 | 0.84 (0.07) | 0.78 (0.06) | .008 |
| AVF (cm$^2$) | 128.0 (67.0) | 56.6 (25.1) | .003 | 68.5 (34.3) | 29.5 (12.8) | .001 |
| ASF (cm$^2$) | 238.1 (61.1) | 172.4 (54.9) | .001 | 303.4 (44.7) | 155.6 (42.4) | <.001 |
| Systolic BP (mm Hg) | 127 (9) | 119 (12) | NS | 125 (16) | 123 (19) | NS |
| Diastolic BP (mm Hg) | 83 (8) | 76 (9) | NS | 82 (13) | 79 (13) | .025 |
| Total cholesterol (mmol/L) | 6.1 (1.1) | 5.8 (1.3) | .017 | 6.4 (1.0) | 5.8 (1.1) | .030 |
| LDL cholesterol (mmol/L) | 4.8 (1.0) | 4.3 (1.1) | .004 | 4.9 (0.9) | 4.3 (1.0) | .013 |
| HDL cholesterol (mmol/L) | 1.2 (0.1) | 1.4 (0.2) | .022 | 1.5 (0.3) | 1.8 (0.5) | .008 |
| Triglycerides (mmol/L) | 1.8 (0.8) | 1.4 (0.2) | .039 | 1.6 (0.4) | 1.1 (0.4) | .021 |
| Fasting plasma glucose (mmol/L) | 5.20 (0.16) | 4.95 (0.15) | .018 | 5.03 (0.14) | 4.77 (0.08) | NS |
| Leptin (ng/mL) | 8.8 (6.2) | 2.8 (2.1) | <.0005 | 20.7 (13.6) | 12.5 (9.1) | .171 |

Values shown are means and SD. Intrapair differences were analyzed by paired-samples *t* test, and logarithmic transformations were used when appropriate. Significance was considered at *P* less than .05. NS indicates not significant.

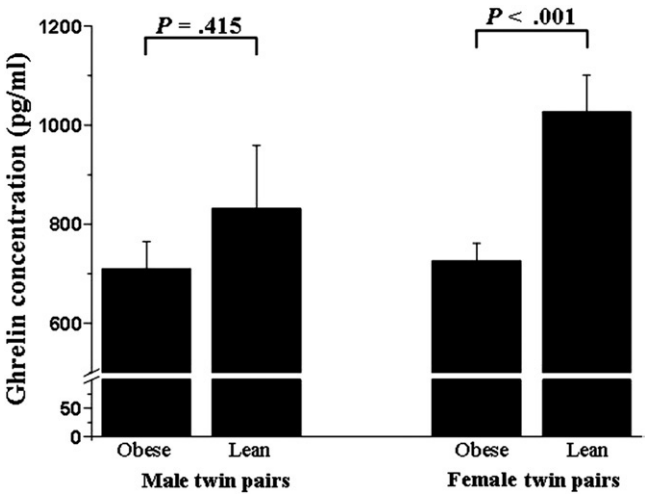


Fig. 1. Fasting plasma total ghrelin concentrations in MZ twins discordant for obesity. There were differences in ghrelin levels between obese and lean twins, with obese co-twins having lower levels than the lean co-twins. The difference was statistically significant in female twin pairs. The statistical intrapair analyses were carried out with paired-samples *t* tests.

Calculated Δ values (differences between obese and lean co-twins) in ghrelin concentrations correlated negatively with those in ASF ($r = -0.573$, $P = .008$) in the whole group. After adjustment for sex in the linear regression model, the correlation disappeared (data not shown). There was no correlation between the intrapair differences in ghrelin concentrations and the intrapair differences in BMI ($r = -0.340$, $P = .113$). There was no correlation between intrapair differences in ghrelin concentrations and those in other anthropometric measures, glucose metabolism indexes, lipids, or leptin levels.

When all study subjects of the obesity-discordant group were considered together, plasma ghrelin concentrations correlated inversely with BMI ($r = -0.484$, $P = .001$), WHR ($r = -0.291$, $P = .050$), AVF ($r = -0.437$, $P = .005$), and ASF ($r = -0.356$, $P = .024$). These correlations were recalculated with sex as a covariate. Body mass index, AVF, and ASF remained significant predictors of the variation in ghrelin concentrations. When sexes were considered separately, in women, ghrelin levels correlated with BMI ($r = -0.638$, $P < .0005$), AVF ($r = -0.450$, $P = .027$), ASF ($r = -0.544$, $P = ,006$), and plasma triglycerides ($r = -0.483$, $P = .009$). In men, these correlations did not reach statistical significance. No correlation between plasma ghrelin and leptin levels was observed.

### 3.2. Genotype frequencies of Agr51Gln and Leu72Met polymorphisms

Most of the subjects were Arg51Arg wild-type homozygotes, only 6 pairs overall were Arg51Gln heterozygotes, and there were no Gln51Gln mutant homozygotes. The Arg51Gln heterozygotes were equally distributed over the groups: 1 pair (4.3%) among the 23 discordant pairs, 2 pairs (4.7%) in the 43 lean-concordant pairs, and 3 pairs (6.5%) in the 46 obese-concordant pairs had the genotype coding for Arg51Gln.

There were differences in genotype frequencies at the Leu72Met polymorphism of the ghrelin gene between all groups ($P = .01$), which could be attributed to differences between the discordant and obese-concordant groups ($P = .003$) and between the discordant and lean-concordant groups ($P = .011$), but not between the 2 concordant groups. The frequency of Met allele was lower in the discordant group (4%) than in the lean and obese-concordant groups (allelic frequencies 15% and 17%, respectively). Genotype and allelic frequencies of the ghrelin Leu72Met polymorphism are shown in Table 2.

## 4. Discussion

Ghrelin levels are decreased in human obesity, but the role of genes in the regulation of ghrelin levels has not been addressed previously. We wanted to begin exploring this issue by determining fasting total ghrelin concentrations in a sample of MZ twin pairs discordant for obesity. The rationale is as follows: because MZ twins share identical genes by descent and yet some pairs exhibit considerable differences in BMI and other obesity markers, would one observe plasma total ghrelin differences between lean and obese co-twin or would a genetic regulation of the hormone level prevail? One of the main findings of this study was that the intrapair difference in total ghrelin concentrations among the obesity-discordant twin pairs was significant, indicating that fasting plasma total ghrelin levels are affected by acquired obesity independent of genetic background. The lean co-twins had higher ghrelin levels than the obese co-twins.

Table 2
Genotype and allelic frequencies of Leu72Met polymorphism in the ghrelin gene among MZ twin pairs

| Twin pairs | Genotype frequency (pairs) | | | Total (pairs) | Allelic frequency (%) | |
|---|---|---|---|---|---|---|
| | Leu72Leu | Leu72Met | Met72Met | | Leu | Met |
| Discordant | 22 | 0 | 1 | 23 | 95.7 | 4.3 |
| Lean concordant | 31 | 11 | 1 | 43 | 84.9 | 15.1 |
| Obese concordant | 31 | 14 | 1 | 46 | 82.6 | 17.4 |
| Total | 84 | 25 | 3 | 112 | 86.2 | 13.8 |

There were differences in genotype frequencies at the Leu72Met polymorphism of the ghrelin gene between all groups ($P = .01$); and in pairwise comparisons, significant differences were seen between the discordant and obese-concordant groups ($P = .003$) and between the discordant and lean-concordant groups ($P = .011$), but not between the 2 concordant groups.

Interestingly, the intrapair difference in ghrelin levels between obese and lean co-twin was seen more clearly among female than male discordant pairs. This might be explained by the larger intrapair difference in BMI, percentage of body fat, and ASF among female pairs. However, as the number of male twin pairs was rather small in our population, these findings only suggest that there may be a sexual dimorphism in the regulation of ghrelin levels. Nonetheless, this population is very unique; the entire Finnish twin cohort of thousands of MZ pairs was screened to find these 23 discordant twin pairs. Hence, undertaking a study with a large sample size will not be a trivial matter.

There was only a trend for a negative correlation between intrapair differences in ghrelin concentrations and BMI, but intrapair differences in ghrelin correlated negatively with intrapair differences in ASF. These findings support the notion that ghrelin levels are affected by obesity. However, no correlation between intrapair differences in ghrelin concentrations and those of other obesity-related parameters such AVF and waist circumference was observed. It is notable that, when correlations were performed with all individual subjects instead of intrapair differences in twin pairs, ghrelin concentrations correlated negatively with all obesity-related parameters, with the exception of leptin levels.

It must be pointed out that ghrelin molecules circulate in bloodstream in various molecular forms differing by their amino acid chain length and by the acyl side chain attached to the third amino acid (serine). Different molecular forms of ghrelin possess different biological effects, for example, on energy metabolism. The most abundant ghrelin molecules of the plasma, namely, the 28 amino acid–long octanoylated and desacylated ghrelins, were measured together (referred to as *total ghrelin*) from the fasting plasma samples in this study. Fasting plasma total ghrelin concentrations correlate well with the diurnal plasma ghrelin concentrations. As our main interest was the regulation of ghrelin levels rather than the biological actions of ghrelin(s), there was no need to measure the acylated and desacylated forms of ghrelin separately.

Monozygotic twins discordant for obesity have the same genes and yet can show large differences in body mass [20,21,33-35]. These differences are due to environmental factors, epigenetic effects, or gene-environmental interactions. One could speculate that discordant pairs carry alleles that are more permissive in the presence of pressures on energy balance, the so-called variability genes. These are in contrast to level genes, where the level of the traits is determined by the presence or absence of specific alleles, a classic example being the effect of the apolipoprotein E polymorphism on lipid values [36]. The latter would be the genes that could account for the apparent discrepancy between the heritability of BMI and obesity in large populations experiencing major increases in the prevalence of obesity. Unless separately and specifically taken into account, gene-environment interactions are subsumed into the estimates of heritability; variability genes would explain in part why some individuals but not others become obese in the current obesogenic environment.

In this present study, there were differences in genotype and allele frequencies at the ghrelin Leu72Met polymorphism between discordant and concordant pairs. The Met allele was significantly more common among concordant twins, suggesting that the ghrelin gene could be one of these "variability genes." The Leu72Leu homozygotes could interact with some unknown factors that make them more vulnerable to environmental factors, possibly related to diet and satiety. Long-term weight change in adults appears to have a genetic component, based both on twin studies [37] and on the Framingham family study [38]. The ghrelin gene polymorphism could be one such factor contributing to the genetic component of weight change in adult life.

In summary, acquired obesity was a more important regulatory factor for fasting plasma total ghrelin levels than the genetic background. The Met allele at the Leu72Met polymorphism was more common among the pairs of MZ twins with low intrapair BMI difference than among those with high intrapair difference. This finding suggests that there may be interactions between sequence variation in the ghrelin gene and unknown environmental factors, thereby supporting the notion that the ghrelin gene may be a variability gene for obesity. Further studies of these issues are clearly warranted using a variety of experimental designs.

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