

RAPID COMMUNICATION

Low serum levels of ghrelin are associated with gallstone disease

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Abstract

AIM: To explore the role of ghrelin in gallstone disease.

METHODS: We carried out a cross-sectional study in 150 subjects, 38 with gallstones (cases) and 112 controls. We also did a real-time PCR-RT study in twenty gallbladder samples each. Body mass index (BMI), serum insulin, ghrelin, and serum lipids were measured. Logistic regression analyses (univariate and multivariate) were conducted to estimate the probability of gallstone disease associated with serum ghrelin concentrations.

RESULTS: Cases were statistically different from controls in gender distribution ($P = 0.01$), age (53 vs 44 yr, $P = 0.002$), BMI (28 vs 25; $P = 0.004$), and glucose (5.26 vs 4.98 mmol/L; $P = 0.05$). The prevalence of ghrelin serum levels above the third tercile was lower in subjects without metabolic syndrome ($P < 0.05$). In a multivariate model, we found a protective effect, when ghrelin values were higher than the median value (OR = 0.27, 95%CI 0.09-0.82, $P = 0.02$). Twenty (20%) gallbladder specimens expressed ghrelin mRNA.

CONCLUSION: Serum ghrelin concentrations are associated with a protective effect of GD.

INTRODUCTION

Human obesity is associated with many significant comorbidities, including cardiovascular disease, diabetes mellitus, hypertension, stroke, osteoarthritis, and some cancers, gallstone disease (GD), *etc*^[1]. GD is exceptionally common in Western populations and is a major source of morbidity in the United States^[2] and Latin American countries^[3]. A relation between obesity and cholesterol gallstones is well recognized. Obese women are more likely to develop gallstones than are obese men^[4]. The bile in obese persons is more lithogenic than is that in nonobese persons, ie, the bile of obese persons has a high ratio of cholesterol to solubilizing lipids (bile acids and phospholipids). This high ratio predisposes to crystallization of cholesterol and gallstone formation. The primary reason for lithogenic bile in obese persons is an increase in total body synthesis of cholesterol^[5,6].

Ghrelin is an endogenous ligand of the growth hormone secretagogue receptor and a potent stimulator of growth hormone release in humans^[7]. Ghrelin is synthesized primarily by the stomach, and in substantially lower amounts by the bowel, pituitary, kidney, placenta, and hypothalamus^[8]. In both rats and humans, the ghrelin gene is made up of 4 exons and 3 introns and the precursors contain 117 amino acids (preproghrelin). Ghrelin processing results in different splicing and/or posttranslational mechanisms^[9]. Two endogenous growth hormones-releasing peptides have been identified, ghrelin and growth hormone-releasing hormone (GHRH) acts on the GHRH receptor to activate adenylate cyclase and

increase intracellular cAMP, which serves as a second messenger to activate protein kinase A. This indicates that the GHRH receptor is coupled to a G_s subunit. On the other hand, ghrelin acts on the GHS-R and activates phospholipase C to generate IP_3 and diacylglycerol, resulting in an increase of intracellular Ca^{2+} , indicating that the ghrelin receptor is coupled to a G_q subunit^[7]

Accumulating evidence suggests that ghrelin contributes to the short- and long-term regulation of body weight as a key element of a complex central signaling network that regulates food intake and energy expenditure^[10]; its concentrations increase preprandially and decrease after meals, suggesting a role in meal initiation. Intravenous administration of ghrelin stimulates food intake in humans^[11] Ghrelin induces a positive energy balance and induces adiposity in rodents by decreasing fat utilization^[12]. Ghrelin concentrations are reported to be decreased in obesity^[13] and increased after diet-induced weight loss^[14].

The aim of this study was to explore the role of ghrelin in gallstone disease.

MATERIALS AND METHODS

Study population

We conducted a cross-sectional study, an immunohistochemistry study and a PCR-RT study in the check-up unit of the Diagnostic Clinic at the Medica Sur Clinic and Foundation (university hospital with subspecialty care) in Mexico City, Mexico. This hospital provides private care for mainly middle- and high-income individuals from Mexico City and the surrounding metropolitan area. Our sample population was formed from a series of consecutive asymptomatic subjects who were referred to the check-up unit by their companies as an annual requirement, not for symptomatic disease. The study included 150 subjects who agreed to participate, 38 subjects found to have GD (25 women, 13 men) and 112 controls (46 women and 66 men without GD). The study was approved by the Human Subjects Committee at The Medica Sur Clinic and Foundation as conforming to the ethical guidelines of the 1975 Declaration of Helsinki, and written informed consent was obtained from all subjects before commencement of the study. All subjects were asked to complete a questionnaire that included demographic and medical variables. The primary demographic and medical variables were gender, age, date of birth, tobacco and alcohol consumption, family history of cardiovascular disease, previous medical conditions, and previous surgery.

Medical exams included an abdominal ultrasonographic study on all subjects as part of the check-up evaluation. Cases were defined as subjects with an ultrasonographic diagnosis of GD (see below), and controls were defined as patients with no such evidence (sensitivity and specificity > 95%)^[15].

Gallstone disease

Abdominal ultrasound was performed using a Sonoline Elegra instrument (Siemens Medical System, Germany) with a 3.5 MHz transducer. Ultrasound diagnosis of GD

was assessed by the presence of strong intraluminal echoes that were gravity-dependent or that attenuated ultrasound transmission (acoustic shadowing). All ultrasonographic studies were evaluated, by the same radiologist on two different occasions. Subjects with previous cholecystectomies were not included in the study.

Diet history questionnaire

Participants completed a food frequency questionnaire with commonly used portion measures specified. The questionnaire included questions on the frequency and brand of multivitamin and individual vitamin supplements. The answers were subsequently electronically scanned and the daily intake of various nutrients was determined using SNUT software, a program developed by the Instituto Nacional de Salud Pública, Mexico City^[16], and appropriate to the Mexican population, in order to estimate dietary energy, protein, carbohydrate, total, saturated, polyunsaturated and monounsaturated fat, vitamin, mineral, and antioxidant intake.

Physical examination

Body weight was measured, in light clothing and without shoes, to the nearest 0.10 kg. Height was measured to the nearest 0.5 cm. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Overweight was defined as a BMI ranging from 25 to 29.9 kg/m² and obesity when BMI was ≥ 30 kg/m² Waist circumference (to the nearest 0.1 cm) was measured at the midpoint between the lower border of the rib cage and the iliac crest, and hip circumference was similarly obtained at the widest point between hip and buttock.

Analytical procedures

Insulin concentrations were measured using an immunoassay (MEIA; Abbott Diagnostics), with inter- and intra-assay coefficients of variation less than 3%.

Serum glucose in the fasting state was measured in duplicate with an automated analyzer. The coefficient of variation for a single determination was 1.5%. Total cholesterol, high-density lipoprotein cholesterol (HDL-C), and triacylglycerol concentrations were measured by enzymatic colorimetric methods, using CHOL, HDL-C plus (second generation) and TG assays (Roche Diagnostics Co., Indianapolis, IN, USA), respectively. Low-density lipoprotein cholesterol (LDL-C) concentrations were calculated using the Friedewald formula^[17].

Assessment of insulin resistance (IR) was made using the Homeostasis Model Assessment (HOMA-IR), as originally described by Matthews *et al.*^[18]: $HOMA-IR = \text{fasting insulin (U/L)} \times \text{fasting glucose (mmol/L)} / 22.5$.

Total serum homocysteine was measured with the fully automated Abbott IMx[®] homocysteine assay (Abbott Laboratories, Abbott Park, IL, USA). Homocysteine is quantified by the intensity of the polarized fluorescent light measured by the IMx optical assembly. Run-to-run CVs ($n = 22$) were 2.9, 0.8, and 1.7% at total homocysteine concentrations of 7, 12.5, and 25 $\mu\text{mol/L}$, respectively.

Serum ghrelin concentrations were determined by radioimmunoassay using RIA kits (Linco Research, St.

Table 1 Demographic, anthropometric, biochemical and diet variables between cases and controls

Variable	Cases		(median min-max)		P-value ¹ Mann-Whitney U test
	n = 38		Controls n = 112		
Age (yr)	52.5	9-80	43.5	18-76	0.002
BMI (kg/m ²)	28.1	17-55	25	19-57	0.004
Body fat (%)	34.1	20-45	27.9	11-47	< 0.0001
Insulin (U/L)	6.2	2-18	5.2	2-51	0.11
HOMA-IR	1.4	0.4-4.8	1.2	0.4-14.1	0.07
Homocysteine (μmol/L)	11.0	5-25	9.0	2-20	0.007
Total cholesterol (mmol/L)	4.99	2.48-8.28	5.07	3-8.51	0.80
HDL-cholesterol (mmol/L)	0.98	0.59-1.97	1.09	0.57-2.02	0.10
LDL-cholesterol (mmol/L)	3.1	1.47-5.43	3.31	0.39-6.28	0.50
Triacylglycerol (mmol/L)	1.6	0.64-4.54	1.42	0.46-3.85	0.18
Energy (kJ)	7624	4743-11417	8323	3043-78803	0.06
Diet proteins (%)	16	12-24	16	10-123	0.93
Diet lipids (%)	31	22-46	34	13-500	0.05
Diet carbohydrates (%)	53	34-64	50	13-68	0.05
Ghrelin (ng/L)	660	129-1267	682	117-1467	0.71
Physical activity (yes) [n (%)]	20 (52.6)		65 (58.0)		0.58 ¹
Metabolic syndrome [n (%)]	15 (39.5)		18 (16.1)		0.006 ¹

¹Fisher's exact test (two-tailed).

Charles, MO, USA). The intra- and inter-assay coefficients of variation were both less than 5%.

Metabolic syndrome

Participants having three or more of the following criteria were defined as having the metabolic syndrome^[9]. The waist to hip ratio > 0.85 and high body fat were defined according to previous definitions^[19].

Ghrelin mRNA expression

Twenty products of cholecystectomy due to symptomatic gallbladder disease were collected prospectively and frozen at -70 °C until the sample was completed. Then, mRNA was extracted with a one-step technique using guanidine isotiocyanate. Complementary DNA was synthesized and processed in real-time PCR using syber green colorant as detector. A qualitative study was performed using β-actin as control.

Statistical analysis

We established a comparison of continuous variables between cases and controls by means of the non-parametric test U of Mann-Whitney due to the non-normality in some variables. Categorical variables compared between cases and controls were evaluated with Fisher's exact test (two-tailed). In order to estimate, as main effect, the probability to be a case (GD) according to serum ghrelin values, we built a multivariate model in a logistic regression analysis, adjusted for potential confounders. The association measure derived from this analysis is the odds ratio (OR) estimate of the exponential of the regression coefficient. The statistical analysis was performed using SPSS/PC v12.0 Software (SPSS, Chicago, IL, USA).

RESULTS

Complete information was obtained in 38 cases and 112

Table 2 Relationship between metabolic syndrome and serum ghrelin levels in subjects with gallstone disease

Ghrelin terciles	Metabolic syndrome criteria					
	0	1	2	3	4	5
1 st	0	1	7	4	1	0
2 nd	1	3	3	0	4	2
3 rd	1	5	2	4	0	0

controls. We observed differences between cases and controls in some variables, particularly in age (cases were older), in anthropometric variables (cases were more overweight, had central obesity and a higher percentage of body fat), and cases had a tendency to have more insulin resistance and higher serum homocysteine values than controls. The median of serum ghrelin values did not show a statistic difference between cases and controls (Table 1).

In addition, there was a statistic difference in the gender distribution between cases and controls. Cases had more women by proportion than controls (65.8% vs 41.1%, $P = 0.01$), more hypertension (42.1% vs 11.5%, $P < 0.0001$) and more metabolic syndrome (39.5% vs 16.1%, $P = 0.006$) (Table 1).

When analyze the relationship between metabolic syndrome and ghrelin serum levels (in subjects with gallstone disease), we observe that in those subjects with metabolic syndrome (three or more criteria, according ATPIII) the prevalence of ghrelin serum levels above the third tercile was lower than in subjects without metabolic syndrome (26.66% vs 34.78%, $P < 0.05$) (Table 2) Similar analysis was applied for subjects without GD, but any significant difference was founded (data not shown).

When we tested the main effect of ghrelin (divided by the median value), a protective association was observed (OR = 0.27, 95%CI 0.09-0.82, $P = 0.02$), indicating a reduction in the probability of being a case subject if

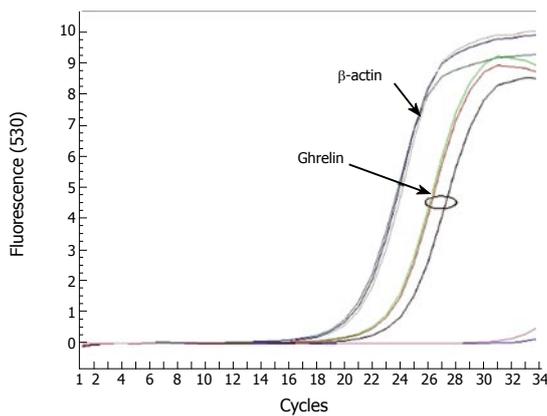


Figure 1 Amplification curves.

the serum ghrelin values are higher than the median, independent of the effect of the control variables: age (yr), gender (males *vs* female), diet lipids (%), homocysteine ($\mu\text{mol/L}$), BMI (kg/m^2), HOMA-IR, and physical activity (yes *vs* no). All the frozen samples showed ghrelin mRNA (Figure 1).

DISCUSSION

In the present study, we found a statistically significant, negative association of ghrelin serum levels and the prevalence of GD. Obesity is the main risk factor for developing GD and insulin resistance, clinically manifested as the metabolic syndrome. The prevalence of obesity has increased worldwide and is closely associated with an increased morbidity and mortality caused by common diseases of the Western world, such as diabetes, hypertension, coronary heart disease, GD and some cancers.^[20] Obese individuals are known to have lower ghrelin serum concentrations than healthy controls. In addition, low concentrations of ghrelin are thought to be a consequence of elevated serum levels of insulin and/or leptin, because fasting serum ghrelin concentrations are negatively correlated with fasting serum concentrations of insulin and leptin.^[13] Recently, ghrelin has been suggested as a regulator of other anorectic cytokines^[21] associated with the metabolic syndrome.

Our results could be interpreted as an effect of the negative correlation between ghrelin serum levels and obesity, considering the burden of obesity in GD. However, after controlling for BMI and HOMA-IR, through multivariate analysis, the association remains statistically significant ($P < 0.05$). Even then, the association could arguably be a product of statistical analysis. However, we believed that there are major facts to be considered.

First, ghrelin was originally described both as a “growth hormone-secretagogue” and a “motilin-related peptide”. Growth hormone has been associated with gallbladder motility, since hypopituitarism is associated with a form of gallbladder dysmotility that resolves after growth hormone replacement^[22] and GD is a frequent complication of long-term octreotide treatment in acromegaly.^[23]

Second, motilin is a well known small intestine-derived

factor in the regulation of gastrointestinal motility in humans involved in receptor-induced gastric motility.^[24] High motilin concentrations can induce growth hormone releasing effects similar to those of ghrelin and high ghrelin concentrations can induce the gastric prokinetic effect of motilin. Luiking *et al* showed that motilin induces gallbladder emptying in humans^[25] and Stolk *et al*^[26] observed an impaired motilin-response in fasting cholesterol gallstones patients.

Third, obesity-related insulin resistance could be a determinant factor in the pathophysiology of GD. Scragg *et al*^[27] demonstrated higher insulin serum concentration in patients with GD, and an associated increase in the bile cholesterol saturation index^[28,29]. In the present study, we observed that cases had lower ghrelin serum levels and higher prevalence of metabolic syndrome, which we have previously reported as a risk factor for GD^[30]. Interestingly, Langenberg *et al*^[31] reported that lower ghrelin levels were associated with a significantly higher prevalence of the metabolic syndrome, with progressively lower ghrelin levels as the number of metabolic syndrome components increased.

Fourth, We demonstrated the expression of mRNA of ghrelin in frozen tissue, suggesting that the gallbladder is capable of producing ghrelin.

In conclusion, our results suggest that serum ghrelin concentrations are associated with a protective effect of GD. We speculate that this is related with a motilin-like effect of ghrelin on the gallbladder motility. However, further studies are needed to determine the exact role of ghrelin in gallbladder physiology.

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