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Circulating follistatin displays a day-night rhythm and is associated with muscle mass and circulating leptin levels in healthy, young humans

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Abstract

Purpose—Follistatin may affect lean and fat mass and be implicated in metabolic diseases. We aimed to elucidate physiological predictors of circulating follistatin variation in healthy young humans.

Procedures—This was an observational, cross-sectional study with two additional prospective observational arms (circadian, seasonal sub-studies) and one prospective interventional arm (mixed meal sub-study). Healthy, young individuals of both sexes (n=122) were subjected to anthropometric and body composition measurements and their eating and exercise behavior profiles were assessed by validated questionnaires. Sub-groups were subjected to standardized meal ingestion (n=36), day-night rhythm (n=20) and seasonal variation (n=20) studies. Main outcome of the study were circulating follistatin levels.

Results—At baseline follistatin levels were correlated with creatinine (r=0.24; p=0.01), creatine phosphokinase (r_s=0.22; p=0.02), and with lean body mass (r_s=0.19; p=0.04) and were higher in males than females (p=0.004) after adjustment for leptin, which was its major predictor. Follistatin levels showed a circadian (p<0.001), but not a seasonal, variation, and were also affected by the

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phase of menstrual cycle in females (p=0.004). Follistatin levels were not affected by dietary or exercise habits but levels increased after a standardized meal ingestion (250 kcal) (p=0.002).

Conclusions—In healthy young individuals circulating follistatin levels are correlated with muscle mass. Follistatin levels are associated with circulating leptin levels and display a day-night rhythm and a menstrual cycle, but not a seasonal, variation.

Keywords

circadian rhythm; fat mass; follistatin; insulin resistance; lean body mass; leptin; seasonal variation

Introduction

Follistatin, a glycoprotein expressed in almost all human tissues, is a known inhibitor of several members of the TGF-beta superfamily, including myostatin and activin [1-3]. Follistatin was initially linked to the reproductive system, as it was discovered in the follicular fluid and found to inhibit follicle-stimulating hormone through activin binding in the pituitary, and was thought to exert mostly autocrine/paracrine actions [4]. Thus, circulating follistatin was considered to originate mainly from such paracrine actions. However, follistatin has been proposed to be an adipokine [5] while recent data suggest an endocrine production from the liver [6] in response to stimuli such as acute exercise [7] or feeding status [8] and the physiological role of follistatin and follistatin-like molecules has expanded to the regulation of fat and lean mass and muscle growth, through activin and myostatin inhibition [2, 9]. Furthermore, follistatin has been implicated in the pathogenesis of muscle atrophy [3] and of metabolic diseases, as it has been reported to be elevated in patients with type 2 diabetes [10], polycystic ovary syndrome (PCOs) [11] and nonalcoholic fatty liver disease (NAFLD) [12]. Thus follistatin may represent an appealing therapeutic target for muscle disorders [13] and metabolic diseases. Nevertheless, the physiological determinants of follistatin and its pattern of secretion remain largely unknown.

The main aim of this study was to evaluate physiologic parameters that could affect circulating follistatin including sex, body composition, eating and activity habits, and meal ingestion. Importantly, we assessed circadian and seasonal variation of follistatin levels and investigated potential correlations with adipokines (leptin, adiponectin) and metabolic parameters.

2. Patients and Methods

2.1. Subjects

This is a post-hoc analysis of a previous clinical study (NCT01986530) [14] with an additional prospective, observational sub-study (Seasonal sub-study). At the initial study, apparently healthy, young Caucasian medical students of both sexes (50% males) were recruited. Exclusion criteria were as previously reported [14]. A sub-group of twenty randomly selected individuals was additionally subjected to seasonal evaluation. The study was approved by the Ethics Committee of 424 General Military Hospital and was in accordance with the Declaration of Helsinki and the International Conference on

Harmonization for Good Clinical Practice. Written informed consent was obtained from all the participants.

2.2. Methods

At baseline, participants had been subjected to anthropometric and body composition measurements and their eating and exercise behavior profiles had been assessed by validated questionnaires, as previously described [14]. Sub-groups of the subjects had participated in the standardized meal ingestion (Fresubin Original Drink Fresenius Kabi, Greece – energy density 1.0 kcal/ml, carbohydrates 55%, protein 15%, fat 30%) and day-night rhythm sub-studies. Morning (8-9 am), fasting venous blood samples had been obtained and metabolic parameters had been measured within 1h after blood drawing using standard methods [14]. Additional samples had been stored at -30 °C. For the purposes of the present study, we conducted additional measurements in stored serum samples. To avoid the interference in the studied parameters of the previous freeze-thaw cycle of the samples, we defrosted a second, unused serum samples obtained from the participants for the new measurements. Subjects participating in the seasonal sub-study where subjected to additional, blood sampling in the middle month of each season, e.g. July, October, January and April. The samples were sent to the laboratory of the Division of Endocrinology at Beth Israel Deaconess Medical Center, Boston, USA at the end of the study for the measurement of follistatin (ELISA, R & D Systems, MN, USA; sensitivity 0.03 ng/ml, intra-assay CV 4.9-7.5%, inter-assay CV 5.2-7.3%), leptin (RIA, Millipore Corporation, MA, USA; sensitivity 0.437 ng/ml, intra-assay CV 3.4-8.3%, inter-assay CV 3.6-6.2%), adiponectin (RIA, Millipore Corporation, MA, USA; sensitivity 1 ng/ml, intra-assay CV 1.8-6.2%, inter-assay CV 6.9-9.3%) and insulin (Immulite 1000, Siemens Healthcare Diagnostics, MA, USA; sensitivity 1 ng/ml, intra-assay CV 5.2-6.4%, inter-assay CV 5.9-8.0%). All study samples were assayed in duplicate in one batch. For the needs of day-night rhythm sub-study, follistatin levels were measured in duplicate twice (with two different kits) to confirm the variation and the average of the two measurements run in duplicate was presented herein. The HOmeostatic Model of Assessment – Insulin Resistance [HOMA-IR = insulin (mU/l) × glucose (mmol/l)/22.5] was used to evaluate insulin resistance.

2.3. Statistical analysis

Data for continuous variables are presented as mean ± standard error of the mean (SEM). Data for categorical variables are presented as numbers and/or percentages. Kolmogorov-Smirnov test was used to check the normality of distribution of the continuous variables. Chi-square test or Fischer's exact test were used to compare categorical variables. Independent samples T-test or Mann-Whitney test were used for between group comparisons, in cases of two groups of continuous variables. One-way analysis of variance (ANOVA) or Kruskal-Wallis test were used in cases of more than two groups of continuous variables. Analysis of covariance (ANCOVA) was used to adjust between group differences for potential confounders. Paired-samples T-test or Wilcoxon Signed Ranks test were used for paired comparisons. Repeated measures ANOVA or Friedman's test were used for more than two paired comparisons. Repeated measures ANCOVA was used to adjust paired measurements for potential confounders. Bonferroni post-hoc correction was used to adjust for multiple pairwise comparisons. Pearson's or Spearman's coefficient was used for

unadjusted binary correlations. Partial coefficient was used for binary correlations adjusted for potential confounders. Multiple linear regression analysis (<<enter>> method) was used to identify variables independently associated with serum follistatin levels. Variables that were not normally distributed were logarithmically transformed for the need of this analysis. A two-sided *p*-value of less than 0.05 was considered statistically significant in all the above tests. Statistical analysis was performed with SPSS 21.0 for Macintosh (IBM Corp., Armonk, NY).

3. Results

3.1. Baseline cross-sectional evaluation of follistatin levels

The descriptive characteristics of the sum of participants included in the baseline cross-sectional evaluation (n=122), including demographic, anthropometric, clinical, biochemical data, as well as dietary and exercise habits, and comparative data between males and females are presented in Table 1, as per our previous publication [14]. Briefly, apparently healthy males (n=61) and females (n=61), aged 20.0 ± 0.1 years and body mass index (BMI) 23.4 ± 0.2 kg/m^2 were recruited. Between males and females, there was no statistically significant difference in follistatin (Table 1). However, after adjustment for leptin, a surrogate for fat mass, estimated follistatin levels were significantly higher in males than females (estimated marginal means: 857 ± 36 and 704 ± 35 pg/ml, respectively; p=0.004). Follistatin levels remained significantly higher in males than females, when waist circumference (estimated marginal means: 856 ± 39 and 705 ± 38 pg/ml, respectively; p=0.015) or BMI (estimated marginal means: 856 ± 39 and 705 ± 38 pg/ml, respectively; p=0.015) or total body fat (estimated marginal means: 861 ± 36 and 701 ± 35 pg/ml, respectively; p=0.004) were added, as confounders of leptin, to the model.

Notably, in females, follistatin levels were lower in the follicular (656 ± 35 pg/ml) than luteal (790 ± 51 pg/ml; p=0.034) phase of menstrual cycle. Regarding dietary habits, circulating follistatin was not significantly associated with the consumption of cereals, vegetables, fruits/nuts, pulses, fish, meat/chicken, dairy or alcohol. Follistatin levels were not also statistically different among the 8 groups of different Med Diet Score (0 to 7; p=0.22) and/or when participants were classified according to the Med Diet Score in a dichotomous manner, i.e., in those with Med Diet Score ≤ 3 and Med Diet Score > 3 (p=0.64). Similar results were observed, when the participants were divided into three groups according to their Med Diet Score (0-2 vs. 3-4 vs. 5-7; p=0.68). Regarding physical activity habits, there was no statistically significant difference in follistatin levels among individuals with low, moderate or high physical activity (p=0.61).

In unadjusted correlations with anthropometric and laboratory parameters, follistatin levels were significantly correlated with creatinine (r=0.24; p=0.01), gamma-glutamyltransferase (GGT; r_s=0.19; p=0.04), creatine phosphokinase (CK, r_s=0.22; p=0.02) and marginally with lean body mass (LBM, r_s=0.19; p=0.04). However, after adjustment for LBM, no correlation remained statistically significant. Moreover, when correlation between follistatin and LBM was adjusted for age or age and gender, it did not remain statistically significant. In multiple linear regression analysis (Table 2), follistatin levels were independently associated with leptin. Based on the sequential models of linear regression analysis (Table 2), the major

determinant of the follistatin was circulating leptin with a standardized beta (β) coefficient ranging between 0.28 and 0.33. Higher leptin levels were associated with higher follistatin levels independently from sex, age, waist circumference, log(HOMA-IR), adiponectin, creatinine, log(GGT) and log(CK). No other variable was independently associated with leptin, in either univariate or sequential multivariate analysis. It is highlighted that leptin was associated with follistatin independently from waist circumference. This remained essentially unchanged when waist circumference was replaced by another measure of adiposity, i.e., BMI or total body fat.

3.2. Mixed meal sub-study

Eighteen individuals received 125 ml and 18 different ones 250 ml of the standardized mixed meal. Age and anthropometric characteristics of either group did not differ statistically from those of the total cohort. Follistatin levels did not significantly change after consuming 125 ml of the standardized meal (baseline 810 ± 72; post-meal 882 ± 93 pg/ml; p=0.13). There was no significant time-sex interaction for the meal of 125 ml (p=0.70). However, follistatin levels were significantly increased after consuming 250 ml of the standardized meal (baseline 734 ± 62; post-meal 936 ± 72 pg/ml; p=0.002).

3.3. Day-night rhythm sub-study

Twenty individuals (10 males) participated in the day-night rhythm sub-study. Follistatin measurements with both kits provided identical results; so their mean values are hereby presented. Circulating follistatin levels revealed a day-night rhythm (*p*-value for trend<0.001; Figure 1). Notably, follistatin displayed two nadir points at 3 pm and midnight and two zenith points at 9 pm and 3 am, with a variation of approximately 44% throughout the day. However, when correction for multiple pairwise comparisons was performed, only point 3 pm showed statistically significant differences vs. 9 am (p=0.003), 9 pm (p=0.025), 3 am (p=0.005) and 6 am (p<0.001). Both males and females showed similar day-night rhythm of follistatin, i.e., biphasic with similar nadir and zenith points (p=0.58 for time-sex interaction). As expected, leptin, used as a positive control, followed the known circadian pattern ((*p*-value for trend<0.001), and insulin-like growth factor-binding protein 3 (IGF-BP3), used as negative control, followed a relatively flat pattern (Figure 1).

3.4. Seasonal sub-study

Twenty individuals (10 males) participated in the seasonal sub-study. There was not statistically significant difference in circulating follistatin levels throughout the year (autumn: 673 ± 47; winter: 758 ± 50; spring: 643 ± 25; summer: 730 ± 71 pg/ml; p=0.31). There was no significant time-sex interaction (p=0.74). Since there was a significant reduction in BMI throughout the year (p=0.017), a time-D(BMI) interaction was also investigated, which, however, did not show a significant interaction (p=0.39).

4. Discussion

Follistatin has been proposed to regulate muscle and adipose tissue growth through activin and myostatin inhibition [1-3, 5]. Thus, follistatin may be implicated in conditions involving muscle and/or fat dysfunction [3, 10]. These conditions, besides the rare myopathies in

young people, also include the sarcopenia [15] and the associated metabolic syndrome of the elderly [16], which tends to become pandemic given the gradual increase in life expectancy not only in the Western countries but worldwide during the last decades [17]. The current lack of approved treatment options for sarcopenia [18] highlights an unmet need. In this concept, follistatin, through inhibition of myostatin and activin, may represent a promising therapeutic target not only for sarcopenia [18] but also for the related metabolic disorders [19] and even inflammation [20]. In the present study, we aimed to identify physiological parameters that could influence circulating follistatin levels. Our data suggest that in healthy, young individuals circulating follistatin is positively correlated with muscle mass and leptin is the major determinant of its levels. There is a day-night but not a seasonal variation. The day of the menstrual cycle influences follistatin levels. Although an acute stimulatory effect of feeding above a certain quantity was observed, dietary and exercise habits do not have a long-term effect on circulating follistatin.

Our findings suggest that in normal state follistatin levels are reflecting muscle mass, as supported by the positive correlation with creatinine, CK and LBM. This is consistent with the role of follistatin to increase muscle mass [2, 3]. This role could be the result of the regulation of follistatin by hormones such as thyroid hormones [21, 22] or other cytokines [23]. In our population, the correlation of follistatin levels with LBM was marginal and was lost after adjustment for BMI. However, LBM reflects muscle and bone mass altogether. Unfortunately we did not measure bone mass and this is definitely a limitation of our study. On the other hand CK and creatinine, which is an indirect index of muscle mass in healthy young individuals, were clearly correlated with follistatin levels, and the correlation for creatinine remained even after adjustment for BMI. After all, in young, lean individuals as our population was, BMI largely reflects muscle mass.

In alignment with the above, the positive correlation we observed between follistatin and leptin levels, in unadjusted models, may reflect an underlying association between leptin levels and overall body mass in young adults. In contrast, in populations with obesity and/or metabolic diseases there might be a dissociation of increasing fat mass from muscle mass which may not keep pace. This hypothesis could explain the decreased follistatin levels reported in obese individuals [5]. An alternative hypothesis for the association between follistatin and leptin could be that, besides muscle, follistatin also promotes adipose tissue increase [5] which could explain the increased levels of follistatin reported in patients with type 2 diabetes [10], PCOs [11] and NAFLD [12]. Furthermore, follistatin may positively affect acute and chronic inflammatory conditions through interaction with inflammatory/immune mediators [20, 24].

We have also found higher follistatin levels in males than females after adjustment for leptin independently from BMI, waist and body fat. Considering the above, this finding is expected, given the higher muscle mass of male participants, the positive association between leptin and follistatin and the lower leptin levels in the males. No difference in follistatin levels between pubertal boys and girls has been reported [25] which is in agreement with the similar levels between sexes we observed before adjusting for leptin.

In our attempt to identify physiologic factors that influence circulating follistatin levels, we found higher levels in the luteal phase of the menstrual cycle in our female participants. Although changes in follistatin expression in the ovaries during the menstrual cycle have been reported in cellular level [26], this is, to our knowledge, the first report of an effect of these changes in the circulating levels of the hormone. This finding has clinical implication in the evaluation of follistatin's normal range in females.

Exercise has been reported to acutely increase plasma follistatin [7]; however, this increase is more likely derived from liver rather than from muscle production [6, 7, 27]. Circulating follistatin levels were not affected by the physical activity habits in this study suggesting that the acute effect is lost in a long-term basis.

Dietary patterns or habits in terms of quantity or quality were also not affecting follistatin levels in our study. On the other hand, feeding exerted an acute quantity-dependent stimulatory effect on follistatin: a standardized meal in two escalating quantities resulted in an increase of its levels which became significant in the higher quantity. This effect could mean that follistatin acts as a mediator by which the consumed glucose and proteins are directed to the muscles to support muscle tissue growth. This might not be the case for consumed lipids, as Hansen et al reported that intralipid infusion resulted in decreased follistatin levels [6]. It has also been previously reported that chronic energy deprivation increases follistatin levels in both healthy males [8] and females [28]. However, acute effects of food intake do not negate a different response to longer term caloric deprivation.

Other findings of our study with clinical implications were the lack of seasonal variation in follistatin levels, which is reported for the first time herein, and their day-night variation with two nadir points at 3 pm and midnight and two zenith points at 9 pm and 3 am. The same pattern was observed in both sexes. No day-night variation has been reported in previous studies in healthy individuals [8, 28], however, these studies included a very small number of subjects which might not be sufficient to reveal changes in follistatin levels. Furthermore, we verified our results by measuring follistatin for a second time using a different kit which exhibited the same variation as the first one. Foster et al [29] have reported circadian rhythm with zenith between 5 and 11 am in girls (mean age 12) with variation of pubertal development and gonadal dysfunction/ovarian failure and adolescents (age 17) with idiopathic hypogonadotrophic hypogonadism. Due to the above controversy, our findings need replication in future, larger studies with more time points of measurement.

Our study has certain strengths. These include the relatively large sample size of apparently healthy individuals, the homogeneity of the population in terms of anthropometric parameters and living conditions, and the thorough investigation of physiological parameters that could influence circulating follistatin levels through validated questionnaires, interventions (standardized mixed meal) and monitoring over time (24h and seasonal variation). A limitation was that our study was cross-sectional, thereby limiting any causative relationship. Another limitation was the fact that body composition was measured by bioelectrical impedance analysis (BIA) rather than dual-energy X-ray absorptiometry (DXA), which is considered to be the gold standard; furthermore, bone mass measurements were not performed.

In conclusion, our data indicate that in healthy, young individuals, circulating follistatin levels are correlated with muscle mass and leptin which is their major determinant. Males have higher follistatin than females after adjustment for leptin. Although a standardized meal exerts an acute quantity-dependent stimulatory effect on follistatin levels, dietary and exercise habits do not have a long-term effect on them. There is a day-night and a menstrual cycle but not a seasonal variation. These data provide important information elucidating follistatin physiology that could also prove useful in the design and performance of future clinical trials.

Contribution Codes / Authors	Conception and design	Acquisition of data	Analysis and interpretation of the data	Drafting of the article	Critical revision of the article	Final approval of the article
Athanasios D. Anastasilakis	☑	☑	☑	☑		☑
Stergios A. Polyzos	☑		☑	☑	☑	☑
Elpida C. Skouvaklidou [3]		☑		☑		☑
Georgios Kynigopoulos		☑		☑		☑
Zacharias G. Saridakis		☑		☑		☑
Aggeliki Apostolou			☑		☑	☑
Georgios A. Triantafyllou			☑		☑	☑
Thomai Karagiozoglou-Lampoudi			☑		☑	☑
Christos S. Mantzoros	☑		☑		☑	☑

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List of abbreviations

BIA bioelectrical impedance analysis

BMI body mass index

CK creatine phosphokinase

DXA dual-energy X-ray absorptiometry

GGT gamma-glutamyltransferase

HOMA-IR HOmeostatic Model of Assessment – Insulin Resistance

IGF-BP3 insulin-like growth factor-binding protein 3

LBM lean body mass

NAFLD nonalcoholic fatty liver disease

PCOs polycystic ovary syndrome

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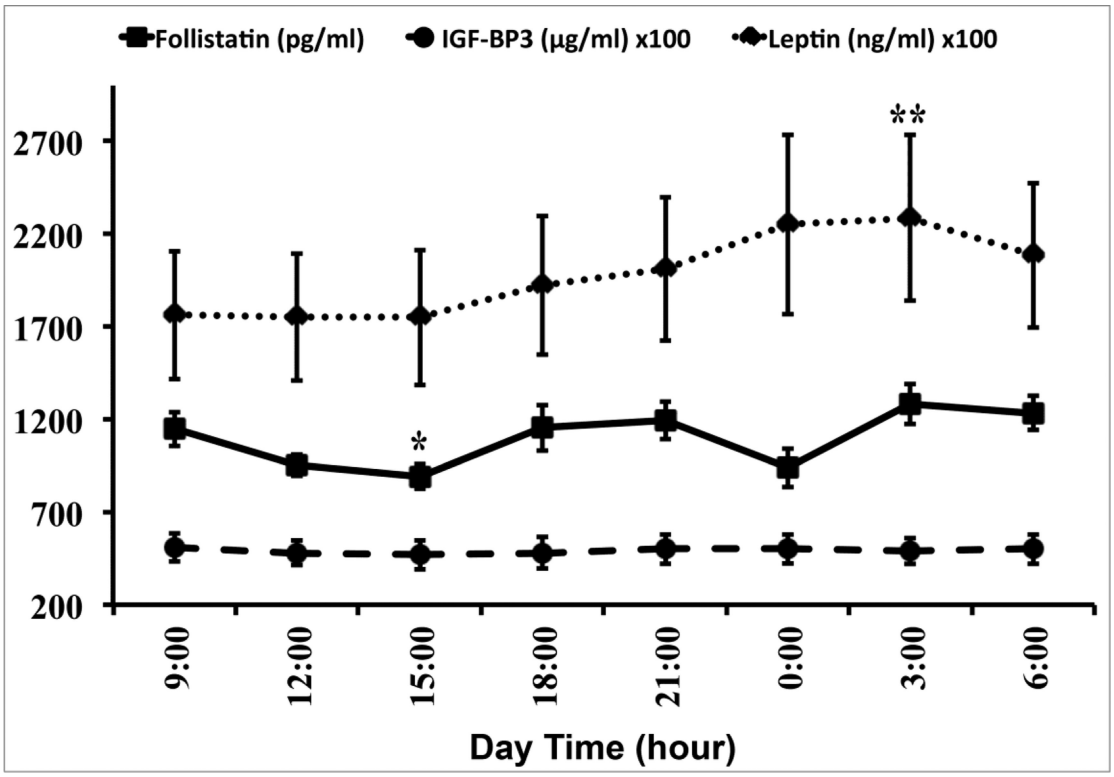

Figure 1.

Day-night rhythm of circulating follistatin, leptin (positive control) and IGF-BP3 (negative control). Data present mean ± standard error of the mean (SEM) for follistatin and leptin, and mean ± standard deviation (SD) for IGF-BP3 (SEMs were too low to appear in the graph).

*: Statistically significant lower levels for time 15:00 vs. 9:00 ($p=0.003$), 21:00 ($p=0.025$), 3:00 ($p=0.005$) and 6:00 ($p<0.001$) (Bonferroni correction for multiple pairwise comparisons).

**: Statistically significant higher levels for time 3:00 vs. 12:00 ($p=0.021$) and 15:00 ($p=0.047$) (Bonferroni correction for multiple pairwise comparisons).

Table 1

Baseline characteristics of all participants and separately of males and females.

	All	Males	Females	*p* value *
N (%)	122 (100.0)	61 (50.0)	61 (50.0)	-
Age (years)	20.0 ± 0.1	20.2 ± 0.1	19.7 ± 0.1	0.01
BMI (kg/cm^2)	23.4 ± 0.2	24.3 ± 0.3	22.5 ± 0.3	<0.001
Waist circumference (cm)	85.7 ± 0.7	88.5 ± 0.9	82.9 ± 1.0	<0.001
Lean Body Mass (kg)	57.5 ± 0.1	67.8 ± 0.9	47.3 ± 0.5	<0.001
Total Body Fat (kg)	13.7 ± 0.7	11.2 ± 1.0	16.2 ± 0.8	<0.001
Follistatin (pg/ml)	779 ± 24	823 ± 37	737 ± 31	0.07
Irisin (ng/ml)	86.6 ± 2.9	91.4 ± 4.8	81.9 ± 3.0	0.11
Leptin (ng/ml)	8.1 ± 0.8	3.9 ± 0.7	12.3 ± 1.3	<0.001
Adiponectin (μg/ml)	15.0 ± 0.5	12.0 ± 0.7	17.9 ± 0.7	<0.001
Glucose (mg/dl)	84 ± 1	85 ± 1	83 ± 1	0.07
Insulin (μIU/ml)	4.5 ± 0.3	5.3±0.5	3.8 ± 0.3	0.02
HOMA-IR	0.95 ± 0.06	1.12 ± 0.10	0.80 ± 0.07	0.01
Creatinine (mg/dl)	0.76 ± 0.01	0.83 ± 0.01	0.69 ± 0.01	<0.001
Total Cholesterol (mg/dl)	161 ± 2	156 ± 4	166 ± 3	0.03
Triglycerides (mg/dl)	59 ± 2	65 ± 3	53 ± 2	0.003
HDL Cholesterol (mg/dl)	56 ± 1	50 ± 1	61 ± 1	<0.001
LDL Cholesterol (mg/dl)	89 ± 2	88 ± 3	90 ± 3	0.62
Aspartate aminotransferase (U/l)	20 ± 1	23 ± 1	17 ± 1	<0.001
Alanine aminotransferase (U/l)	17 ±1	20 ± 2	13 ± 1	<0.001
GGT (U/l)	14 ± 1	16 ± 1	12 ± 1	<0.001
Alkaline phosphatase (U/l)	65 ± 2	75 ± 2	56 ± 2	<0.001
CK (U/l)	205 ± 31	292 ± 47	122 ± 38	<0.001
Smoking (current or ex-; N%)	6 (4.9)	4 (6.6)	2 (3.3)	0.68

Data are presented as mean ± standard error of the mean (SEM) for continuous variables and frequencies for categorical variables.

Abbreviations: CK, creatine phosphokinase; GGT, Gamma-glutamyltransferase; HOMA-IR, homeostasis model of assessment insulin resistance; HDL, High Density Lipoprotein; N, number; LDL, Low Density Lipoprotein.

* p-value for the between gender comparisons.

Table 2

Multiple linear regression analysis evaluating independent associates of serum follistatin levels.

Variable	β	Standardized β	*p*-value	95% CI for β
Sex (0: males; 1: females)	−25.8	−0.05	0.79	−206.1 – 154.6
Age (years)	44.0	0.15	0.12	−11.7 – 99.7
Leptin (ng/ml)	9.1	0.31	0.007	2.6 – 15.6
Waist circumference (cm)	2.2	0.07	0.54	−5.1 – 9.6
Log(HOMA-IR)	−94.7	−0.10	0.32	−280.9 – 91.3
Adiponectin (μg/ml)	−1.2	−0.03	0.81	−11.1 – 8.7
Creatinine (mg/dl)	590.5	0.23	0.08	−74.2 – 1255.2
Log(GGT) (mg/dl)	130.8	0.07	0.51	−261.3 – 522.9
Log(CK) (U/l)	67.8	0.09	0.41	−93.3 – 228.8

Abbreviations: CK, creatine phosphokinase; GGT, gamma-glutamyltransferase; HOMA-IR, homeostasis model of assessment insulin resistance