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Relationships between Acylated Ghrelin and Parameters of Metabolic Profile in Patients with Non-Alcoholic Fatty Liver Disease Depending on Transaminases Activity.

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ABSTRACTS

Ghrelin is a hormone produced mainly by the cells lining the fundus of the stomach, which is involved in regulation of lipid and glucose metabolism. The aim was to explore the level and relationships of acylated-ghrelin AG in non-alcoholic fatty liver disease (NAFLD) patients depending of transaminase activity. In this cross-sectional study, 91 type 2 diabetes (T2D) patients were included. All patients were divided into 3 groups. The control group included 28 T2D patients without NAFLD. The main group included 63 T2D patients with NAFLD, which was divided in 2 subgroups depending on transaminase levels: normal (n=37) and elevated (n=26) transaminases group. We observed 1.5 (p=0.016) and 2.5 (p<0.001) fold increasing of serum AG levels in patients with NAFLD and normal or elevated transaminases compared to control groups. AG level associated with BMI in all study groups. In normal transaminase group AG significantly correlated with TG, VLDL-C and insulin, but after sex, gender and BMI adjusting significant association stayed only for TG (r=0,294, p=0,048). AG in NAFLD patients with elevated transaminases significant associated only with ALT (r=0,445; p=0.023) and AST (r=0,497; p=0.010). Our study has demonstrated that elevated AG level were associated with NAFLD.
Keywords: non-alcoholic fatty liver disease, acyl-ghrelin, type 2 diabetes

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) ranges from simple steatosis to non-alcoholic steatohepatitis (NASH) with/without cirrhosis, and hepatocellular carcinoma [1,2]. The increased prevalence of type 2 diabetes (T2D), obesity, hypertension and dyslipidemia in the general population is considered to be the most common cause for NAFLD [3]. The improved management of chronic viral hepatitis, have resulted in NAFLD becoming a leading cause of chronic liver disease and a major health concern owing to hepatic and extrahepatic morbidity/mortality [4,5]. The pathogenesis of NAFLD can be explained as the 'two hit' theory [6]. Insulin resistance (IR) as the key mechanism provokes overload of hepatocytes by free fatty acids and lead to development of hepatic steatosis thought to represent the 'first' step [7]. The "second hit" is a consequence of oxidative stress in hepatocytes that promotes the inflammation, fibrosis and necrosis associated with NASH [8,9].

Ghrelin is a 28 amino-acid peptide with an n-octanoyl group at the serine 3 residue, produced mainly by the stomach, which was identified as the endogenous ligand of the growth hormone secretagogue receptor. The preproghrelin gene-derived peptides include acyl (AG), dys-acyl ghrelin (DAG), and obestatin. The major circulating form that constitutes to 80–90% of circulating ghrelin is DAG [10]. Yang et al. [11] identified enzyme which implicated in the n-octanoylation of ghrelin, namely GOAT (Ghelin O-Acyltransferase) that is expressed in the major ghrelin-secreting tissues. Data from animal studies suggested that ingestion of medium-chain fatty acids increased the stomach concentrations of AG without changing the total (acyl- and des-acyl-) ghrelin amounts [12]. Also both AG and DAG, in differentiating omental adipocytes, significantly increased PPAR-γ and SREBP1 mRNA levels [13] all of which are positive modulators of hepatic triglycerides contents by targeting genes coding for key reactions in lipid *de novo* synthesis. Consequently, both the ghrelin forms may play a role in excessive fat accumulation in obesity and thereby NAFLD. The earlier human studies were performed by total ghrelin assays and suggested that in obesity, IR and T2D it level were decreased [14,15]. Therefore, the aim of this study was to explore the level and relationships of AG in NAFLD patients depending of transaminase activity.

MATERIALS AND METHODS

Study subjects

In this cross-sectional study, 91 T2D patients with age of 40–80 years from the Kyiv City Clinical Endocrinology Center were selected. Inclusion criteria were: age over 18 years, presence of T2D in association with or without NAFLD. NAFLD diagnosis was concluded according to the recommendations of the American Gastroenterology Association (AGA) and American Association for the Study of Liver Disease (AASLD) on the basis of: clinical examination, laboratory values of lipid and carbohydrate metabolism, liver enzyme activities (ALT, AST), ALT/AST ratio, and ultrasonography examination [16]. Exclusion criteria included alcohol abuse (>210 grams of alcohol per week in men and >140 grams of alcohol per week in women over a 2-year period), chronic viral hepatitis (associated with HBV, HCV, HDV infection), drug-induced liver disease, Wilson's disease, hereditary deficiency of antitrypsin-1 and idiopathic hemochromatosis. The ethics committee of Kyiv City Clinical Endocrinology Center approved the study.

Data collection and measurements

After informed consent, fasting serum samples were obtained and immediately frozen at −80°C. For each patient, relevant clinical and demographic data were collected. Anthropometric data including weight and height were measured to the nearest 100 g and 0.5 cm, respectively. Body mass index (BMI) was calculated as body weight in kilograms divided by the square of the participant's height in meters.

Plasma total cholesterol (TC), HDL-cholesterol (HDL-C) and triglyceride (TG) concentrations were measured using enzymatic kits, standardized reagents and standards (BioVendor, Czech Republic). LDL-cholesterol concentration was calculated using the Friedewald equation [17]. Blood glucose was determined using the Trinder's glucose oxidase method while serum insulin was measured with the double radioimmunoassay (RIA) method (AIA-Pack IRI; Tosoh, Tokyo). Insulin resistance was assessed by the validated homeostasis model assessment (HOMA) index [18] using the following formula: HOMA-IR=(FPG * FPI)/22.5, where FPG and FPI are fasting plasma glucose (mmol) and fasting plasma insulin (μU/ml), respectively.

The diagnosis of fatty liver was based on the results of abdominal ultrasonography, which was done by trained technicians with Ultima PA (Radmir Co., Kharkiv, Ukraine). Of 4 known criteria (hepatorenal echo contrast, liver brightness, deep attenuation, and vascular blurring) [19], the participants were required to have hepatorenal contrast and liver brightness to be given a diagnosis of NAFLD.

Fasting serum AG concentrations were measured from plasma samples stored at −20°C using a commercial "peptide enzyme immunoassay" kit (Peninsula Laboratories, Inc., California, USA) with intra-assay and inter-assay variations of coefficients (CV) of less than 5% and 14%, respectively, sensitivity limit of 0.08-1.0 ng/ml, and measuring range of 0-25 ng/ml. No cross-reactivity was found with human DAG, motilin, secretin, human vasoactive intestinal peptide, human prolactin-releasing peptide-31, human galanin, human growth hormone releasing hormone, neuropeptide Y, orexin A, orexin B.

Statistical analysis

The SPSS statistical package, version 20.0 (SPSS, Inc., Chicago, Illinois) was used for all statistical analyses and a *P* value less than 0.05 was considered statistically significant. All continuous values are expressed as mean±SD and categorical variables are presented as %. Data distribution was analyzed using the Kolmogorov-Smirnov normality test. Continuous variables with parametric distribution were then analyzed using one-way ANOVA and if the results were significant, a Bonferroni Post Hoc test was performed. Data with non-parametric distribution was analyzed using Kruskall-Wallis test. For comparisons of categorical variables we conducted χ^2 test.

Univariate and multivariate linear regression analyses were used to identify relationship between AG and clinical, anthropometric and laboratory parameters. Variables which are statistically significant in univariate analysis were included in the stepwise multivariate linear regression analysis. Backward stepwise selection was used at a stringency level of $p<0.10$ to detect the independent risk factors on NAFLD. A $p<0.05$ probability level was considered as statistically significant.

RESULTS

All patients were divided on the next groups. The control group included 28 (30.76%) T2D patients without NAFLD (mean age was 53.57±7.16, T2D duration 3.5±1.57). The main group included 63 (69.24%) T2D patients with NAFLD, which was divided in 2 subgroups depending on transaminase levels: normal transaminases group included 37 (58.73%) patients (mean age was 53.27±8.39, T2D duration 5.97±3.88 years) and respectively elevated transaminases group included 26 (41.27%) patients with mean age 51.5±10.92 and T2D duration 8.54±5.57 years.

Table 1: Anthropometric, clinical and laboratory parameters in examined patients (M±SD)

Parameters	Control (n=28)	p1	p2	NAFLD with normal transaminases (n=37)	NAFLD with elevated transaminases (n=26)	p3	P
Age, years	53.57±7.16	-	-	53.27±8.39	51.5±10.92	-	NS
Duration of T2D, years	5.0±2.81	-	-	5.97±3.88	8.54±5.57	-	NS
BMI, kg/m^2	31.15±3.0	0.028	<0.001	34.46±5.64	40.35±5.63	<0.001	<0.001
ALT, U/L	25.03±7.26	NS	<0.001	28.93±6.15	64.96±14.99	<0.001	<0.001
AST, U/L	23.91±6.11	NS	<0.001	26.6±5.71	55.8±11.91	<0.001	<0.001
FPI, μIU/ml	11.21±3.58	0.001	<0.001	18.27±8.14	20.61±8.56	NS	<0.001
FPG, mmol/l	7.80±1.41	-	-	8.67±3.25	9.48±3.14	-	NS
HOMA-IR	3.86±1.41	<0.001	<0.001	6,86±3.21	8.23±2.85	NS	<0.001
TC, mmol/l	5.68±0.68	0.004	<0.001	6.24±0.72	6.56±0.62	NS	<0.001
TG, mmol/l	1.83±0,45	0,018	<0,001	2,65±1,2	3,36±1,55	NS	<0,001
VLDL-C, mmol/l	0.82±0.2	0.005	<0.001	1.25±0.59	1.39±0.63	NS	<0.001
HDL-C, mmol/l	1.66±0.25	0.001	<0.001	1.43±0.24	1.2±0.26	0.002	<0.001
LDL-C, mmol/l	3.18±0.6	0.047	<0.001	3.61±0.69	4.07±0.78	0.034	<0.001

p - difference between all study groups calculated using one-way ANOVA
p 1-3 - for pairwise comparisons used Bonferroni Posthoc test
p1 - the difference between the control and normal transaminases groups
p2 - the difference between the control and elevated transaminases groups
p3 - the difference between normal and elevated transaminases groups

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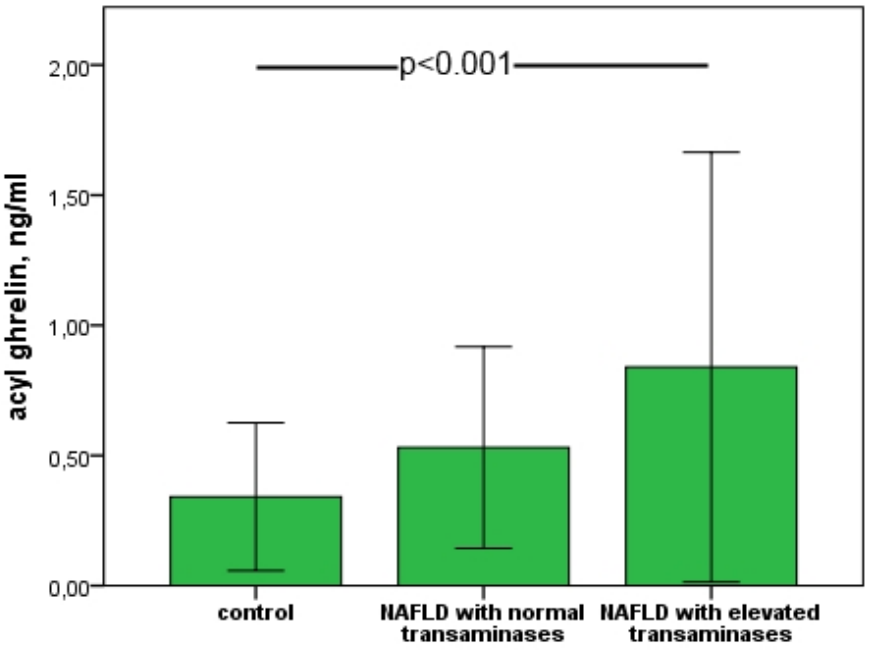
Results of anthropometric, clinical and laboratory parameters are represented in the Table 1. There was no statistically significant difference in the T2D duration between all study groups (p=0.068). The longest duration of T2D was observed in patients with NAFLD and elevated transaminases. BMI was elevated in all patients. We found significant increasing of BMI parallel to NAFLD presence and it severity. The prevalence of obesity (defined as BMI>30.0) in control group was 64.3%, while in NAFLD patients the rate were higher: 75.7% for normal transaminases and 100% for elevated transaminases group (p=0.004) respectively. Morbid obesity (BMI>40.0) was diagnosed only in patients with NAFLD (16.2% vs 46.2%, p<0.001).

In all patients we found violation of the carbohydrate metabolism. Mean values of HOMA-IR and insulinemia were higher in NAFLD patients with maximum on elevated transaminases groups (p<0.001). The value of HOMA-IR exceeding 3 (indication of IR) were found in 78.6% of patients from control group, in NAFLD groups, depending of transaminase activity, we determine IR in 94.6% and 96.2% patients respectively (p=0.048).

Dyslipidemia was present in all study patients. We observed growth of TC (p<0.001), TG (p<0.001), VLDL-C (p<0.001) and LDL-C (p<0.001) parellel with the development of NAFLD and activity of transaminase. While HDL-C levels decreased. Highest mean lipid levels were observed in patients with NAFLD and elevated transaminases.

Level of serum AG was significantly higher in patients with NAFLD compared to the control group (fig. 1). We also mentioned significant difference between mean AG values in NAFLD patients depending on transaminase activity (0.53±0.19 vs 0.83±0.41, p=0.001).

Figure 1: Serum AG levels in study patients



In univariate Pearson correlation analysis we established that AG level associated with BMI in all study groups (fig 2-4). In control group the strength of link between this parameters was (r=0,394; p=0.018) the weakest as compared to normal (r=0,453; p=0.005) or elevated (r=0,457; p=0.019) transaminases groups.

In control group after adjusting on sex, age and BMI we additionally established association bettwen AG level and HOMA-IR (r=0,469; p=0.018) and hyperinsulinemia (r=0,421; p=0.036) (fig. 2, table 2).

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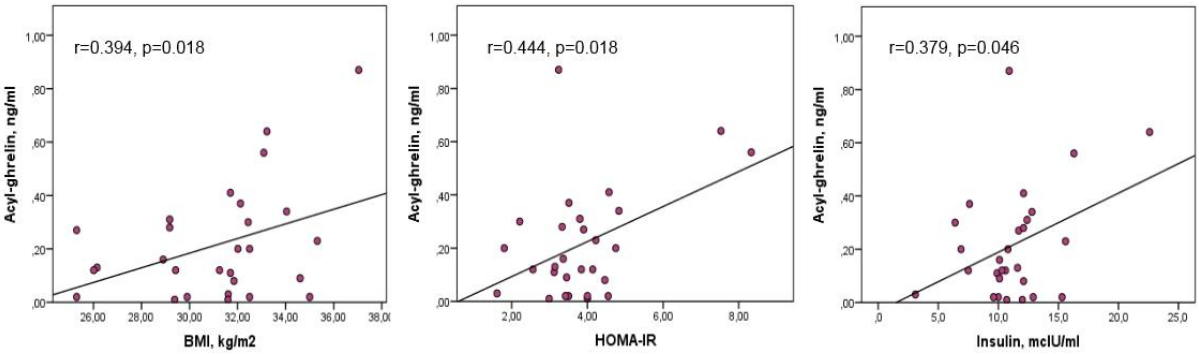
Table 2: Partial correlation analysis of AG with anthropometric, clinical and laboratory parameters.

Parameters	Control		NAFLD with normal transaminases		NAFLD with elevated transaminases	
Age	0.226 (0.247)	-	0.216 (0.199)	-	-0.125 (0.542)	-
Gender	0.113 (0.565)	-	0.303 (0.068)	-	0.018 (0.931)	-
BMI	0.394 (0.038) *	-	0.453 (0.005)*	-	0.457 (0.019) *	-
Duration T2D	-0.079 (0.689)	-0.177 (0.398)	0.263 (0.115)	0.310 (0.075)	0.173 (0.398)	0.136 (0.536)
ALT	0.149 (0.450)	0.081 (0.702)	0.109 (0.521)	0.080 (0.651)	0.445 (0.023) *	0.429 (0.041) *
AST	-0.109 (0.480)	-0.208 (0.319)	0.250 (0.135)	0.372 (0.089)	0.497 (0.010) *	0.423 (0.044) *
Insulin	0.379 (0.046) *	0.421 (0.036) *	0.373 (0.023) *	0.285 (0.102)	-0.020 (0.924)	-0.056 (0.799)
HOMA-IR	0.444 (0.018) *	0.469 (0.018) *	0.251 (0.134)	0.162 (0.360)	0.351 (0.078)	0.279 (0.198)
Cholesterol	-0.270 (0.290)	-0.134 (0.523)	0.219 (0.193)	0.235 (0.182)	0.359 (0.072)	0.260 (0.232)
TG	-0.214 (0.274)	0.043 (0.839)	0.420 (0.010) *	0.294 (0.048) *	0.162 (0.428)	0.244 (0.262)
HDL	-0.062 (0.573)	-0.123 (0.557)	-0.197 (0.243)	-0.034 (0.847)	-0.180 (0.379)	-0.189 (0.387)
LDL	-0.138 (0.484)	-0.107 (0.611)	0.025 (0.882)	0.030 (0.867)	0.250 (0.218)	0.076 (0.732)

The data are presented as r (p).
*- noted statistically significant correlation

Figure 2: Significant determinants which associated on univariate Pearson's correlation analysis with serum AG level in control group.



In normal transaminase group AG significantly correlated with TG (r=0,420, p=0,010), VLDL-C (r=0,452; p=0,006) and insulin (r=0,373; p=0.023) (fig 3), but after sex, gender and BMI adjusting significant association stayed only for TG (r=0,294, p=0,048) (table 3).

Table 3: Stepwise multiple linear regression analysis using as dependent variable serum ghrelin level.

Models	Unstandardized coefficients	SE	β	p
Control (adjusted R^2 =0,266) Constant HOMA-IR BMI	 -0.781 0.061 0.025	 0.364 0.025 0.012	 0.409 0.354	 0.021 0.043
NAFLD with normal transaminases Model 1 (adjusted R^2 =0,294) Constant BMI TG Model 2 (adjusted R^2 =0,238) Constant TG Insulin	 0.832 0.034 0.145 -0.025 0.151 0.019	 0.426 0.012 0.056 0.215 0.059 0.009	 0.401 0.362 0.379 0.326	 0.008 0.015 0.014 0.033
NAFLD with elevated transaminases (adjusted R^2 =0,312) Constant BMI ALT	 -2,462 0.055 0.020	 0.999 0.022 0.008	 0.414 0.400	 0,021 0,025

SE – standard error of unstandardized coefficients,
R^2– determination coefficient.

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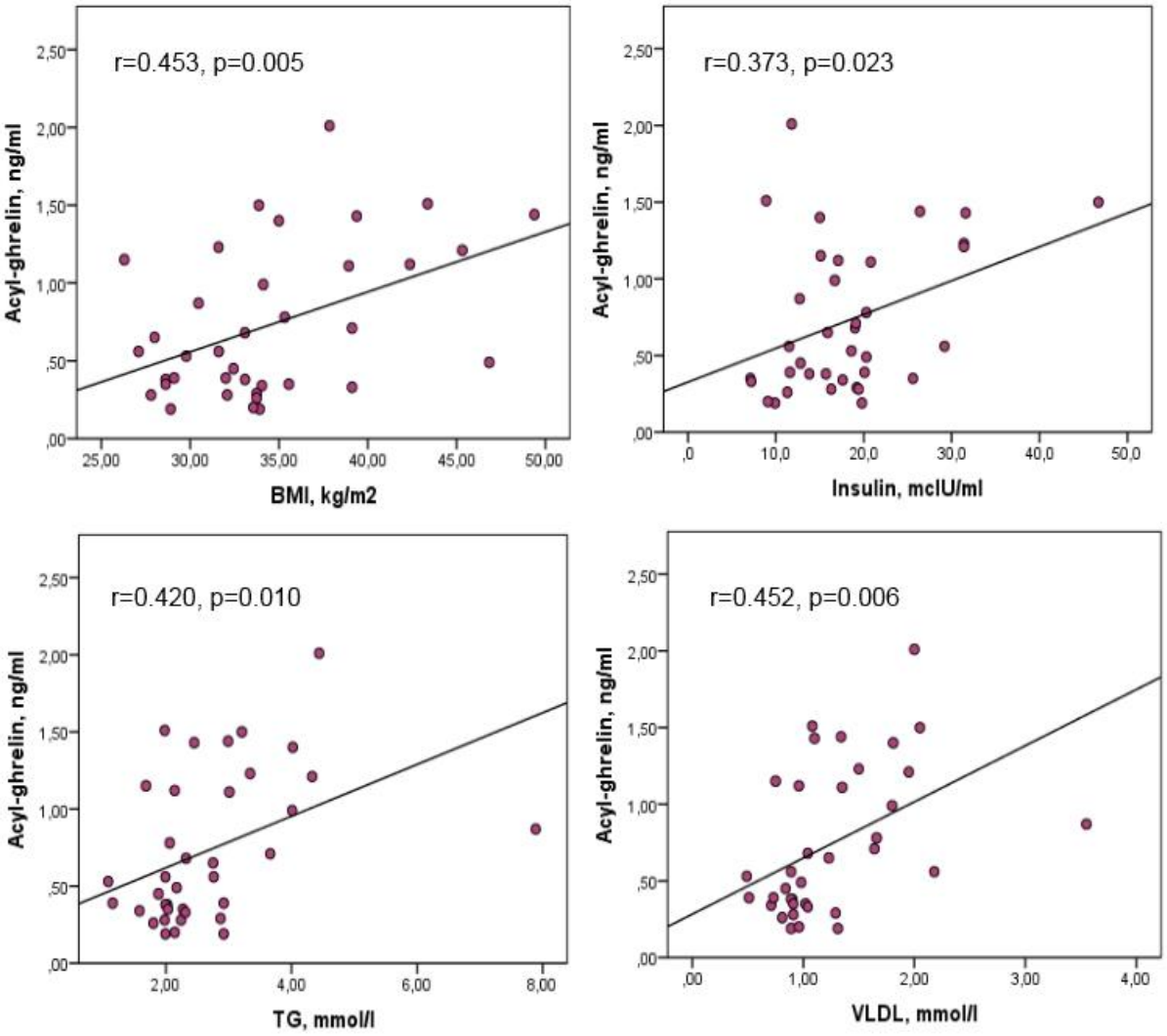
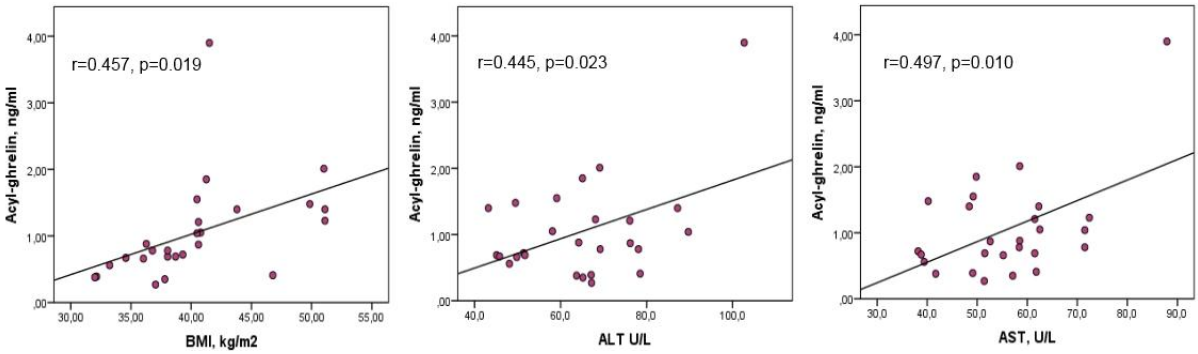
Figure 3: Significant determinants which associated on univariate Pearson's correlation analysis with serum AG level in normal transaminases group.



AG in NAFLD patients with elevated transaminases significant associated except of BMI only with ALT (r=0,445; p=0.023) and AST (r=0,497; p=0.010) (fig 4, table 3).

Figure 4: Significant determinants which associated on univariate Pearson's correlation analysis with serum AG level innormal transaminases group.



All variables which were statistically significant in univariate analysis were included in the stepwise multivariate linear regression analysis. As seen in Table. 3 we constructed several regression models for predicting serum AG concentration.

DISCUSSION

NAFLD has emerged as the most common cause of chronic liver disease worldwide. The most widely supported theory of NAFLD pathogenesis implicates IR as the key mechanism leading to accumulation of triglycerides in the hepatocytes [6, 7]. Therefore fatty liver develops when *de novo* synthesis exceed the oxidation and re-secretion of TGs and the most important mechanism which lead to this are increased *de novo* lipogenesis [20, 21] as a result of over-expression of the transcription factors PPAR-γ [22], ChREBP and SREBP-1c [23], all of which are positive modulators of hepatic TG. Recent study showed that incubation with both AG and DAG, in differentiating omental adipocytes, significantly increased PPAR-γ and SREBP1 mRNA levels, as well as several fat storage-related proteins, including acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), lipoprotein lipase (LPL) and perilipin [13]. Consequently, both the ghrelin forms may play a role in excessive fat accumulation in obesity and thereby NAFLD.

Gutierrez-Grobe et al. [24], in cross-sectional study included 98 subjects (51 NAFLD patients and 47 controls), reported that compared to control NAFLD patients had significantly lower level of serum ghrelin. In multivariate logistic regression analyses high ghrelin (OR=0.128, 95CI 0.048-0.343, p<0.001) level had a protective effect against hepatic steatosis after controlling for potential confounders. Other available data mentioned a negative association between total ghrelin and BMI or insulin levels [14, 15, 25], but role of different ghrelin forms are still contradictory.

The balance AG/DAG and relative AG excess may negatively modulate insulin action in obese patients, thus contributing to the association of IR with NAFLD. Rodriguez et al. [13.] found that AG levels were increased, whereas DAG levels were decreased in obesity. Although lean volunteers showed ~3% of AG and ~97% of DAG, in obese individuals, the relative amounts changed to ~10% in the acylated form and ~90% in the desacylated form of the hormone. Furthermore, AG values were higher in obese individuals with impaired glucose tolerance and T2D than in obese normoglycemic patients. No effect of glucose tolerance or diabetes was observed in circulating DAG concentrations. A highly significant positive correlation was observed between AG and BMI, WC, and HOMA, whereas DAG showed a strong negative correlation with these parameters. Additionally AG inversely associated with TC, HDL-C, LDL-C and DAG with transaminases activity. Langenberg et al. in large cohort of patients with metabolic syndrome demonstrated that systolic BP and diastolic BP, fasting and postchallenge insulin, HOMA-IR, HDL-C and triglycerides were associated with total ghrelin before and after adjustment for age and sex. Adjustment for BMI attenuated most associations; only HDL-C remained significantly associated with ghrelin [26].

St-Pierre et al. in a group of 89 non-diabetic overweight or obese postmenopausal women found that the dysregulation of ghrelin secretion profiles during a euglycemic/hyperinsulinemic clamp (EHC) is associated with IR. They determined whether insulin-sensitive overweight or obese (ISO) and insulin-resistant overweight or obese (IRO) individuals display different AG and DAG profiles during EHC. DAG levels were found to be significantly decreased for ISO and IRO individuals during the EHC, whereas only ISO subjects displayed a significant reduction of AG levels ($p<0.05$). Insulin sensitivity was significantly correlated with maximal reduction of AG ($r=0.36$; $P < 0.05$) concentrations [27].

Recent study [28] on 45 adult patients with metabolic syndrome mentioned that plasma insulin and HOMA-IR were negatively associated with total ghrelin and DAG, but positively with AG and the AG/DAG ratio in 33 obese compared to 12 non-obese patients. This data in accordance with our study, were we found significant association between AG and HOMA-IR only in patients with T2D and obesity. Therefore circulating ghrelin profiles, and a relative AG excess or DAG deficiency can contribute to obesity-associated IR.

In recent study Marchesini et al. suggested that compared to 40 matched healthy subjects, patients with NAFLD (n=86) had reduced level of total ghrelin. In relation to quartiles of HOMA-IR, ghrelin decreased in both groups, and significantly correlated with HOMA-IR. After adjustment for age and sex, HOMA-IR was the sole factor significantly associated with low ghrelin (below 235 pmol/l) in the whole group (OR 5.79; 95% CI 2.62–12.81; p<0.0001) and specifically in NAFLD (OR 2.96; 95%CI 1.12–7.79; p=0.028). Authors suggest that IR is a major factor controlling total ghrelin levels in subjects with and without NAFLD [29].

We reported 1.5 (p=0.016) and 2.5 (p<0.001) fold increasing of serum AG levels in patients with NAFLD and normal or elevated transaminases compared to control groups. Our data in concordance with the study of Estep et al [30]. They reported, in 75 morbidly obese patients with biopsy-proven NAFLD (41 with histologic NASH), that circulating AG concentrations in NASH with fibrosis score ≥2 were almost double the concentration of NASH patients with a fibrosis stage <2 (8.73 vs. 4.22 pg/ml, p<0.04). But no significant differences in AG levels between NASH and patients with non-NASH (6.42±15.0 vs 2.85±6.0, p>0.05) authors did not found.

CONCLUSION

In conclusion, our data indicate that relative AG excess is associated with BMI in patients with NAFLD and T2D. Depending of transaminases activity we found significant correlation between AG and TG, HOMA-IR and functional state of the liver. Furthermore investigations will be needed to elucidate whether dysregulation of ghrelin secretion profiles in NAFLD patients may influence the long-term metabolic outcomes.

Abbreviations

AGA, American Gastroenterology Association; AASLD, American Association for the Study of Liver Disease; AG, acyl-ghrelin; ALT, Alanine transaminase; ANOVA, Analysis of Variance; AST, Aspartate transaminase; BMI, Body mass index; DAG, dys-acyl ghrelin; EHC, euglycemic/hyperinsulinemic clamp; FFA, free fatty acids; FPG, fasting plasma glucose; FPI, fasting plasma insulin; IR, insulin resistance, IRO, insulin-resistant overweight; ISO, insulin-sensitive obese; HDL-C, high density lipoprotein; HOMA, homeostasis model assessment; LDL, low density lipoprotein; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OR, odds ratio; TC, total cholesterol; TG, triglyceride; VLDL-C, cholesterol of very low density lipoproteins;

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