Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2022 Feb 12; 10(B):470-476.
https://doi.org/10.3889/oamjms.2022.7548
eISSN: 1857-9655
Category: *B - Clinical Sciences*
Section: *Endocrinology*

Diagnostic Value of Acyl-Ghrelin in Type 2 Diabetic Patients with Non-alcoholic Fatty Liver Disease

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Edited by: Slavica Hristomanova-Mitkovska
Citation: Youssef EM, El-Beik DEDMS, Bayoumy EM, Saleh OM, Ezzat WM, Siddik RI, Mohamed AE, Sayed MM, Ali MO. Diagnostic Value of Acyl-Ghrelin in Type 2 Diabetic Patients with Non-alcoholic Fatty Liver Disease. Open Access Maced J Med Sci. 2021 Feb 12; 10(B):470-476. https://doi.org/10.3889/oamjms.2022.7548
Keywords: Acyl-Ghrelin; Nonalcoholic fatty liver; Type 2 diabetes mellitus; Diagnosis
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Received: 09-Oct-2021
Revised: 15-Nov-2021
Accepted: 02-Feb-2022

Funding: This research did not receive any financial support
Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Nonalcoholic fatty liver disease (NAFLD) has become the leading cause of chronic liver disease worldwide. Type 2 diabetes (T2D) is described as one of the most significant risk factor for developing NAFLD, non-alcoholic steatohepatitis, and advanced cirrhosis. Liver biopsy cannot be used routinely to diagnose NAFLD. Therefore, it is critically urgent to develop a simple non-invasive test.

AIM: This study examined fasting Acyl-Ghrelin (AG) as a non-invasive biomarker to accurately diagnose NAFLD in diabetic patients.

PATIENTS AND METHODS: Sixty-one patients with T2D were divided into a test group with NAFLD, and a control group without NAFLD. Secondary causes of fatty liver, chronic viral hepatitis, and drug-induced liver damage were excluded from the study. Anthropometric measurements, lipid profile, fasting blood sugar (FBS), liver enzyme activities, and fasting AG levels were collected. Data management and analysis were performed using statistical package for social sciences version 20.

RESULTS: Fasting AG level (pg/ml) in the test group (56.1 ± 10.7) was increased, but not statically significant compared with the control group (37.8 ± 9.3), $p > 0.05$. However, significant metabolic changes were observed in body weight, waist circumference, FBS, alanine transaminase, and aspartate transaminase between test and control groups. The mean values in the test group are 93.2 ± 14.5, 115.4 ± 7.6, 144.2 ± 25.9, 21.1 ± 5.7, and 32.3 ± 2.1. While the mean values are 87.7 ± 7.3, 95 ± 3.8, 123.7 ± 20.7, 18.6 ± 5, and 20 ± 7, respectively, in the control group.

CONCLUSIONS: While elevated AG levels alone were not significant, elevated AG levels plus other parameters of liver damage and obesity were associated with the diagnosis of NAFLD. However, more studies are needed to consider elevated AG as a diagnostic marker in NAFLD patients with T2D.

Introduction

Nonalcoholic fatty liver disease (NAFLD) can broadly be defined as conditions caused by excessive fat accumulation in hepatocytes not promoted by excessive alcohol consumption. Nearly, 20% of NAFLD patients develop non-alcoholic steatohepatitis (NASH) which leads to liver cirrhosis or failure and even hepatocellular carcinoma [1]. NAFLD is strongly linked to several risk factors, the presence of which influences the disease severity and progression [2]. Type 2 diabetes mellitus (T2D) and metabolic syndrome are known to be the most important risk factors for NAFLD development, two-fold increased risk of incident T2D [2], [3].

As a Middle Eastern country with a population of over 100 million people, Egypt is ranked among the top ten nations in the world in terms of obesity and T2D. NAFLD was found in 57.65% of an obese Egyptian adolescent cohort [4]. 20% of people who had NAFLD develop NASH. Moreover, 20% of those eventually develop cirrhosis as an advanced stage of liver disease [3], [4]. The prevalence of NAFLD and its consequences are expected to rise shortly and become a serious public health burden, and therefore, there is an urgent need for the development of a simple and non-invasive diagnostic test for NAFLD in individuals at high risk [5], [6], [7].

It is now well established from a variety of studies that imbalances between liver lipid output and input are the most important components in fatty liver development. The main factors influencing this energy disturbance are: (1) Increased systemic free fatty acid (FFA) and insulin resistance (IR) as a result of increased adipose tissue lipolysis; (2) increased dietary fat consumption as a source of FFAs; (3) increased hepatic de novo lipogenesis; (4) change in lipoprotein production or secretion; (5) Decreasing fatty acid β-oxoxidation in mitochondria [8], [9], [10]. The cellular shift in metabolism from fatty acid oxidation to *de novo* lipid synthesis is regulated by the activity of the three known transcription factors peroxisome proliferator-activated receptor gamma (PPAR-γ),

carbohydrate response element-binding protein, and Sterol-regulatory element-binding protein-1C (SREBP-1c) [11], [12], [13].

Ghrelin, a novel peptide hormone of 28 amino acid residues, is produced by the stomach as well as other organs such as the liver and gallbladder. It showed significant effects on hepatic fatty acid metabolism in some experimental models [14]. According to Chen *et al*. in 2004, exogenous ghrelin is thought to stimulate appetite and food consumption through induction of hypothalamic neuropeptide Y/agouti-related peptide neurons expressing GHS-R type 1a [15]. Moreover, ghrelin affects energy metabolism, lipid, and glucose homeostasis, at the peripheral level by modulating insulin secretion and sensitivity in pancreatic b-cells and increasing glucose output by primary hepatocytes [16], [17], [18], [19]. Among the peptides generated from the preghrelin gene are Acyl-Ghrelin (AG), Des-AG (DAG). Interestingly, both ghrelin and DAG appear to have many functions in addition to the direct control of food intake, among them is the development of hepatosteatosis and its progression to NASH [20], [21], [22], [23]. Thus, this study evaluated AG as a diagnostic non-invasive biomarker for the detection of NAFLD in diabetic patients.

Patients and Methods

This cross-sectional study was conducted on 61 T2D patients in association with or without NAFLD over 5 months from April 2021 to August 2021. Before the study was conducted, ethical approval (code number 1451022021) and informed written consent from the participants had been obtained. Demographic and clinical data then were collected at the time of consultation through a pro forma completed by a qualified physician at the internal medicine clinic in the National Research Centre. Patients with hepatitis B virus antigen or hepatitis C virus antibody and patients with chronic liver disease were excluded from the study.

The participants' age falls between the ages 25 and 73. Twenty-nine of the diabetic patients without NAFLD were considered as control, 13 (45%) of them were female, while 16 (55%) were male. Thirty-two of the diabetic patients were diagnosed by abdominal ultrasonography and were considered as the test. Of those 26 (81%) were female, while 6 (19%) were male. Ultrasonographic measurements were performed by experienced radiologists based on 4 known criteria (hepatorenal echo contrast, liver brightness, deep attenuation, and vascular blurring). The participants were required to have hepatorenal contrast and liver brightness to be given a diagnosis of NAFLD.

Anthropometric measurements and laboratory tests were carried out included weight (kg), height (cm), and waist circumference (cm). Body mass index (BMI) (kg\m^2) was calculated by dividing the weight in kilograms by the height in meters squared. Blood pressure (BP) was measured twice, with the subjects in a sitting position, using an automated device; the mean of 2 measurements was calculated.

Blood samples were collected after a fasting period of at least 12 h, plasma was separated by centrifugation for the following laboratory investigations:

Liver function tests were including the activity levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) using the colorimetric assay from Bio diagnostic and Research Reagents Company, Dokki, Egypt. Fasting blood sugar (FBS) using enzymatic colorimetric assay from Bio diagnostic and Research Reagents Company, Dokki, Egypt. Lipid profile, the concentrations of total cholesterol (Tc), high-density lipoprotein (HDL), and triglycerides (TG) were measured using colorimetric enzymatic assays. Low-density lipoprotein (LDL) concentration was calculated using the Friedewald formula. Some of the plasma samples were immediately frozen at −80°C for the AG test by ELISA technique from Sino gene clon Co., Ltd. For Statistical analysis: Data were coded, entered, and analyzed using statistical package for social sciences version 20. The quantitative variables were compared using paired t-test or one-way analysis of variance. Qualitative variables were compared using the Chi-square test or Fisher's exact test. p-value level of significance: $p > 0.05$: non-significant, $p < 0.05$: significant, and $p < 0.01$: highly significant.

Results

Table 1 and Figure 1 below show that age, sex, pulse, and BP did not differ significantly between T2D patients with NAFLD and control. The statistical test used is tow sample t-test.

Table 1: Demographic parameters in the examined patients (M ± SD)

Demographics	Control (T2D) n = 29 M ± SD	T2D with NAFLD n = 32 M ± SD	p-value
Age	45 ± 7	48 ± 10	0.252
Range	33–68	25–73	
Sex	16 (55.2%)	6 (19%)	0.0071
Male	13 (44.8%)	26 (81%)	0.005
Female			
Pulse\min	79 ± 4.5	80 ± 3.9	0.214
Blood pressure	125 ± 12.5	127 ± 15.6	0.434
Systolic (mm Hg)	77 ± 8.4	80 ± 10.2	0.298
Diastolic (mm Hg)			

p ≤ 0.05 significant, NAFLD: Nonalcoholic fatty liver disease, T2D: Type 2 diabetes.

A significant increase ($p < 0.05$) in waist circumference in T2D with NAFLD group was recorded compared with the control. BMI was elevated also in T2D with NAFLD patients but no significant difference between the two groups was evident.

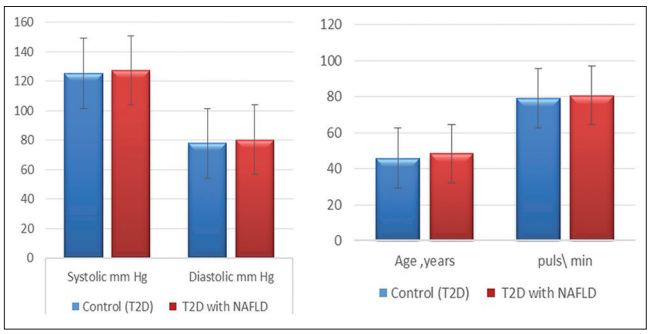

Figure 1: Demographic parameters in the examined patients

The mean values of body weight, length, waist circumference, and BMI are 87.7 ± 7.3, 158 ± 6.3, 95 ± 3.8, and 34 ± 4.2, respectively, for the control group, and in T2D with NAFLD group are 93.2 ± 14.5, 165.4 ± 7.9, 115.4 ± 7.6, and 37 ± 5.1 (Table 2).

Table 2: Anthropometric parameters in the examined patients (M ± SD)

Anthropometric Parameter	Control (T2D) n = 29 M ± SD	T2D with NAFLD n = 32 M ± SD	p-value
Body weight (kg)	87.7 ± 7.3	93.2 ± 14.5	0.087
Length (cm)	158 ± 6.3	165.4 ± 7.9	<0.001
Waist Circumference (cm)	95 ± 3.8	115.4 ± 7.6	0.0354
BMI (kg/m^2)	34 ± 4.2	37 ± 5.1	0.1

p ≤ 0.05 significant, NAFLD: Nonalcoholic fatty liver disease, T2D: Type 2 diabetes, BMI: Body mass index.

In addition, significant increases of FBS, TG, LDL, ALT, and AST levels were found in T2D with NAFLD group compared with the control group; however, no significant differences in TC level between groups.

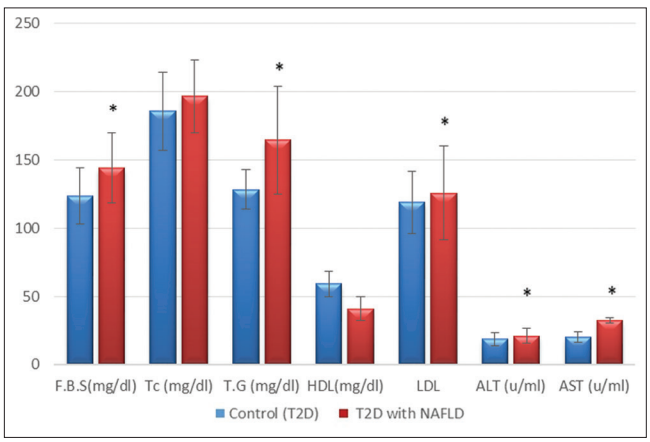

Figure 2: Biochemical parameters in the examined patients (M ± SD)

Table 3: Biochemical parameters in the examined patients (M ± SD)

Parameters	Control (T2D) n = 29 M ± SD	NAFLD with T2D n = 32 M ± SD	p-value
FBS (mg/dl)	123.7 ± 20.7	144.2 ± 25.9	0.035
TC (mg/dl)	185.7 ± 28.6	196.7 ± 26.8	0.299
TG (mg/dl)	128.4 ± 14.4	164.5 ± 39.4	0.004
HDL (mg/dl)	59.3 ± 9.3	40.9 ± 8.5	0.288
LDL (mg/dl)	119 ± 22.9	125.8 ± 34.6	<0.0001
ALT (u/ml)	18.6 ± 5	21.1 ± 5.7	0.016
AST (u/ml)	20 ± 4	32.3 ± 2.1	0.01

p ≤ 0.05 significant, NAFLD: Nonalcoholic fatty liver disease, T2D: Type 2 diabetes, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, FBS: Fasting blood sugar, TC: Total cholesterol, HDL: High-density lipoprotein, TG: Triglycerides, LDL: Low-density lipoprotein.

The mean of FBS, TC, TG, HDL, LDL, ALT, and AvST are 123.7 ± 20.7, 185.7 ± 28.6, 128.4 ± 14.4, 59.3 ± 9.3, 119 ± 29, 18.6 ± 5, and 20 ± 4, respectively, for the control group, and in T2D with NAFLD group are 144.2 ± 25.9, 196.7 ± 26.8, 164.5 ± 39.4, 40.9 ± 8.5, 125.8 ± 34.6, 21.1 ± 5.7v and 32.3 ± 2.1 (Table 3 and Figure 2 and 3).

Table 4: Fasting acyl-ghrelin in the examined patients (M ± SD)

Parameter	Control (T2D) n = 29 M ± SD	NAFLD and T2D n = 32 M ± SD	p-value
Fasting acyl ghrelin (pg/ml)	37.8 ± 9.3	56.1 ± 10.7	0.306

p ≤ 0.05 significant, NAFLD: Nonalcoholic fatty liver disease, T2D: Type 2 diabetes.

A clear increment was observed in fasting AG level in T2D with NAFLD group; however, it was not significant compared with the control group (Table 4 and Figure 4).

Table 5: Stepwise multiple linear regression analysis using the dependent variable fasting acyl- ghrelin level

Model	Regression coefficient ± SE	OR (95% CI)	p-value
(Nagelkerke $R^2 = 0.7443$)			
Constant	12.23 ± 9.326		
Weight (kg)	0.502 ± 0.37	1.098 (1.018–1.214)	0.02
Length (cm)	0.296 ± 0.42	0.9136 (0.826–0.9991)	<0.0001
FBS (mg/dl)	0.546 ± 0.35	1.026 (1.001–1.062)	0.024
TG (mg/dl)	0.394 ± 0.39	0.9691 (0.937–0.9938)	0.003
LDL (mg/dl)	0.451 ± 0.38	0.9746 (0.951–0.9934)	0.018
ALT u/ml	0.077 ± 0.095	1.08 (0.898–1.319)	0.4141
AST u/ml	0.582 ± 0.34	0.886 (0.718–0.9937)	0.036

The statistical test used: Tow sample t-test, *P*≤0.05 significant (95% confidence interval), AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, FBS: Fasting blood sugar, TG: Triglycerides, LDL: Low-density lipoprotein.

In Table 5, only ALT did not show a significant correlation while all other parameters showed significant correlations with fasting Acyl- Ghrelin.

Table 6: Diagnostic accuracy of fasting acyl-ghrelin for NAFLD diagnosis and differentiation between patients with elevated and normal values

Parameter	NAFLD versus control
Cut-off value	<0.26
Sensitivity, %	48.2
Specificity, %	60
NPV, %	9.4
PPV, %	93.1
AUROC	0.569
95% confidence interval	P-value
p-value	0.3553

p ≤ 0.05 considered statistically significant (95% confidence interval), NAFLD: Nonalcoholic fatty liver disease, T2D: Type 2 diabetes, NPV: Negative predictive value, PPV: Positive predictive value, AUROC: Area under the receiver operating characteristic curve.

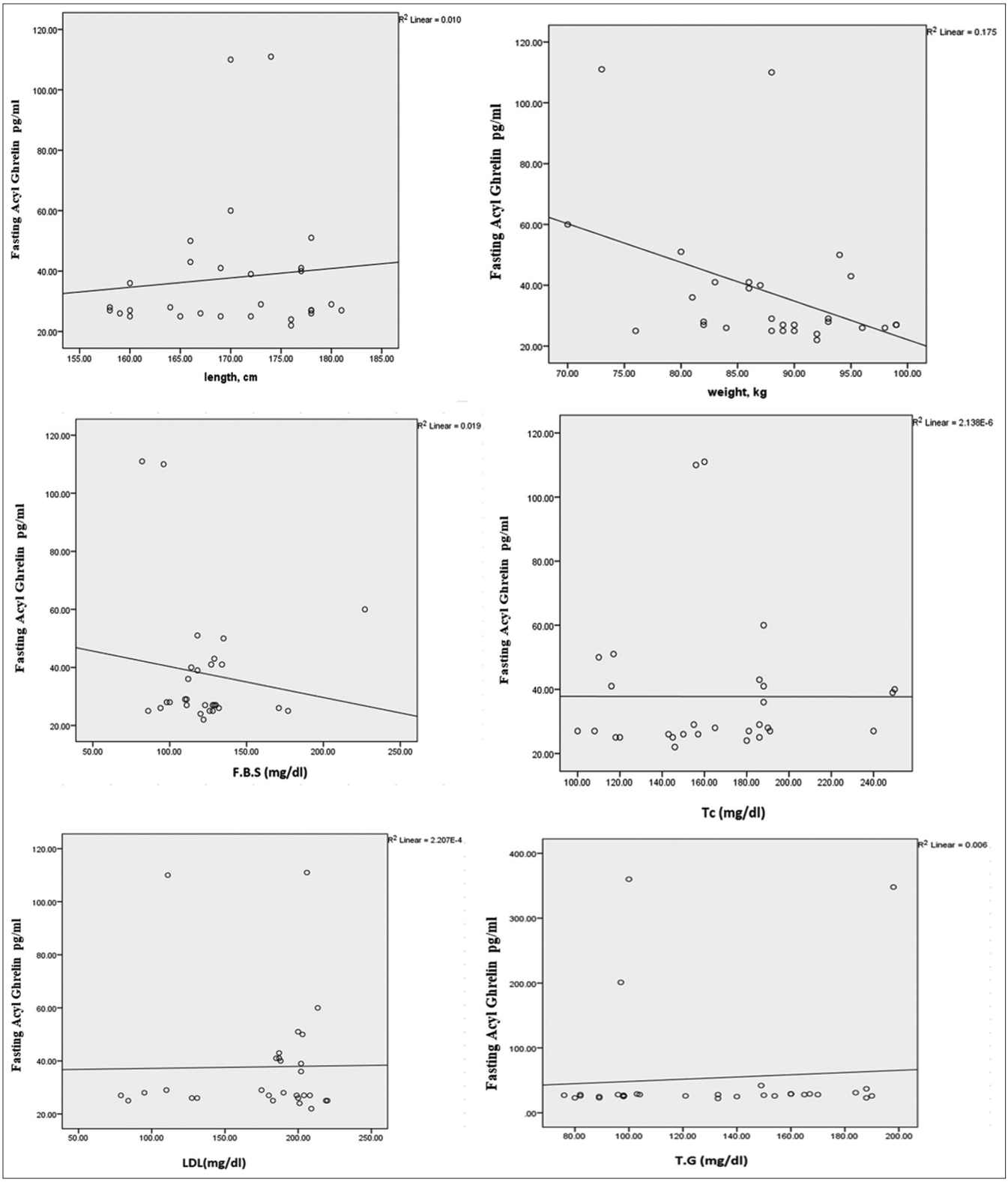

Figure 3: Significant determinants which associated on univariant Pearson's correlation analysis with serum acyl ghrelin level in nonalcoholic fatty liver disease group

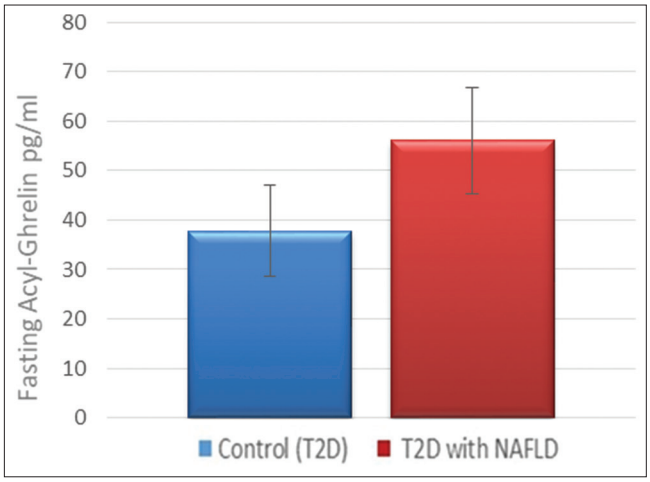

Figure 4: Fasting acyl ghrelin in the examined patients (M ± SD)

Table 6 and Figure 5 show a low accuracy level of using AG test to identify NAFLD condition in T2 D patients.

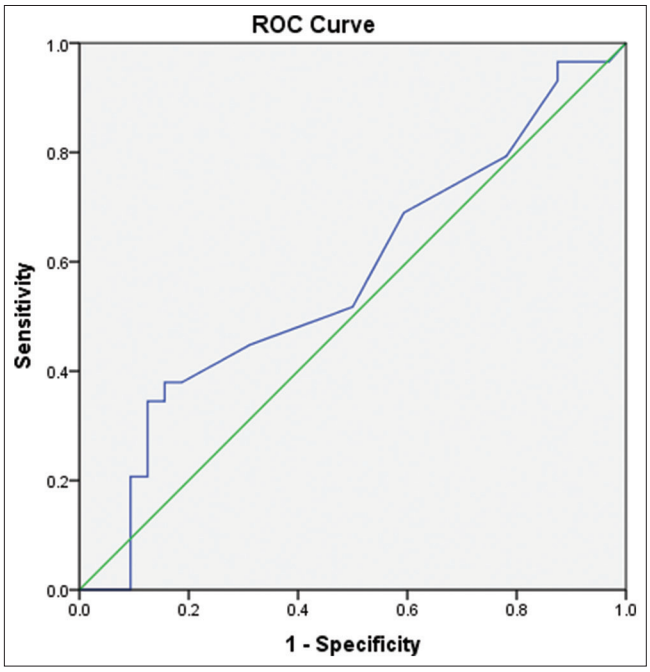

Figure 5: Receiver operating characteristic curve

Discussion

NAFLD, the common cause of liver-related morbidity and mortality, is a growing public health concern worldwide [1], [2], [23]. However, several studies have reported that T2D patients are expected to be at a higher risk of developing liver disease including fibrosis, cirrhosis, and hepatocellular carcinoma compared with healthy populations [24], [25]. Until recently, there has been little interest in safe, accurate methods to screen for NAFLD in T2D patients especially in populations with a high prevalence of NAFLD. Moreover, a large proportion of type 2 diabetics are diagnosed with NAFLD long after the onset of their diabetes which makes it difficult to determine the duration and the risk of developing NAFLD [3], [26].

In this regard, our study aimed to assess the possibility of using fasting AG hormone as a reliable noninvasive marker of NAFLD in diabetic patients. The rationale for the measurement of AG is based on the known effects of ghrelin on hepatic fatty acid metabolism observed in previous experimental models and recently proposed as a link in the development of NAFLD in patients with T2D [27], [28].

In line with these observations, our data showed elevations of fasting AG levels that differed between the NAFLD and control T2D group. Although this difference was not statistically significant, AG levels were positively correlated with FBS, TC, TG, HDL-cholesterol (HDL-C), LDL cholesterol, and AST. These findings are in agreement with those observed in recent studies of Mykhalchyshyn *et al*. and others [28], [29], [30], [31].

Neuman *et al*. and Kraft *et al*. partly explained these relationships in various tissues through a well-proven mechanism of AG action [30] in which AG was found to stimulate both lipogenesis and gluconeogenesis, raise TG levels, and reduce fatty acid oxidation-stimulating activity. The impact of AG on TG deposition was noted to be greater in the liver than skeletal muscle [29], [31].

Our findings in terms of a relation between hyperglycemia and elevated AG in NAFLD with T2D cases reflect those of Broglio *et al*., and Dezaki *et al*. [32], [33]. They referred it to the direct action of ghrelin on pancreatic α- and β-cells which results in the secretion of glucagon, and glucose-induced secretion of insulin, respectively. This increases hepatic glucose production and decreases glucose uptake and insulin sensitivity in skeletal muscle and adipose tissue, and causes elevated blood glucose levels [34], [35].

In addition, the observed positive correlations between body weight, waist circumference, and elevated AG level in T2DM patients with NAFLD are in line with those of previous studies obtained by Liu *et al*. and Neuman *et al*. [30], [31]. They suggested that AG could cause IR and promote liver fat deposition through a core hypothalamic mechanism. PPAR-g and SREBP1, as well as other fat storage-related proteins including acetyl-CoA carboxylase, lipoprotein lipase, and fatty acid synthase, are the most important factors [17], [21], [33] in the development of NAFLD. Moreover, the elevated FFA in obese subjects can result in IR and subsequent increase in ghrelin levels in NAFLD patients [34], [35]. On the other hand, acyltransferase, an enzyme involved in the n-octanoylation of ghrelin, namely ghrelin O-acyltransferase is another factor that may explain the correlation. Obviously, obesity influences the expression and/or activity of acyl transferase which causes elevation of AG plasma concentrations [36].

This outcome is contrary to that reported by Ukkola *et al* and Amini *et al*., who found that plasma ghrelin was negatively associated with fasting glucose levels in

T2D patients [37], [38]. Furthermore, Özcan *et al*., found that low total ghrelin concentrations were associated with some features of metabolic syndrome including elevated BP, hypertriglyceridemia, and obesity [39].

In the present study, low levels of HDL-C and high levels of TG, the key features of IR-associated dyslipidemia, were significantly different between patients with and without NAFLD. These findings broadly support the work of other studies in this area linking NAFLD with metabolic syndrome [37], [38], [39]. Significant increases in waist circumference, and FBS concentration in T2D with NAFLD patients are in line with the results of previous studies obtained by Rahimi *et al*., and Portillo *et al*. who reported an association of liver fat content with increased insulin requirements which have the potential to fuel weight gain [40], [41].

Based on the literature which have reported the involvement of AG in the pathogenesis of NAFLD, in the present study, we hypothesized that AG may have potential as a NAFLD biomarker in diabetic patients. At the present time, the results of AG levels alone were found not to be significantly associated with NAFLD in diabetic patients. The reasons for this are not clear, but possible causes include high variances as reflected by high standard deviations of AG. Another limitation is that the number of available control cases for the diabetes without NAFLD is low. Unfortunately, there are only limited number of published mechanistic studies on the role of AG in energy homeostasis and obesity in NAFLD. However, there are data that indicate that both factors affect AG responsiveness.

Conclusions

The findings of this study provide insights on the role of AG in the development of NAFLD. However, validation of the use of AG as a serum marker of NAFLD in T2D patients needs further studies with a large number of patients.

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