

Pro-Neurotensin as a Potential Novel Diagnostic Biomarker for Detection of Nonalcoholic Fatty Liver Disease

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Background and Aims: Currently, liver biopsy is the gold standard method for diagnosis of non-alcoholic fatty liver severity. It is critical to develop non-invasive diagnostic method to diagnose nonalcoholic fatty liver rather than invasive techniques. Our case-control study was to address the value of circulating miRNA-122 and serum pro-neurotensin as a potential non-invasive biomarker for the diagnosis of non-alcoholic fatty acid diseases.

Methods: Clinical assessment, laboratory investigations, and anthropometric measurements were reported for 157 patients with proven NAFLD. Apparently, healthy participants (n=100) were enrolled as a control group. Serum samples were tested for micro-RNAs-122 and pro-neurotensin.

Results: Compared with the control subjects, both mi-RNA-122 and serum proneurotensin levels were increased in NAFLD ($p<0.001$) and at a cut-off ≥ 6.83 , mi-RNA-122 had 51.0% sensitivity, 70.0% specificity to differentiate NAFLD from healthy controls, while serum proneurotensin had 80.0% sensitivity and 80.0% specificity at a cutoff ≥ 108 .

Conclusion: The circulating pro-neurotensin might be used as a novel biomarker for diagnosis of patients with NAFLD, wherefore the integration of a circulating mi-RNA-122 and serum pro-neurotensin could be beneficial to diagnose NAFLD cases. Large-scale studies are needed to investigate the possible role of mi-RNA-122 and pro-neurotensin in the development, progression, and prognosis of NAFLD and NASH.

Keywords: nonalcoholic fatty liver disease, NAFLD, metabolic associated fatty liver disease, MAFLD, biomarker, proneurotensin, micro-RNA-122, noninvasive, diagnosis, fatty liver

Introduction

Nonalcoholic fatty liver disease (NAFLD) is considered one of the health problems with great burden on the liver that may end with liver cirrhosis and hepatocellular carcinoma.¹ Universally, nonalcoholic fatty liver disease (NAFLD) has a prevalence of 24% and is considered as a high risk of liver-related morbidity and mortality,² which has been described as the most important precursor to hepatocellular carcinoma (HCC).³ NAFLD is characterized by the macrovesicular accumulation of triglycerides in the liver cells, leading to necro-inflammation and fibrosis, and may progress to cirrhosis and hepatocellular carcinoma. HCC is considered the fifth most abundant type of tumor worldwide and the third most lethal cancer, causing 600,000 deaths each year.^{4,5} Ultrasound is the most widely used imaging test for NAFLD diagnosis, but its sensitivity decreases if $<30\%$ of the liver is affected by steatosis. Additionally, computed tomography

(CT scan) is accurate for diagnosing moderate-to-severe liver steatosis; however, it is not accurate for detecting mild steatosis.⁶

Liver biopsy is the gold standard invasive procedure for diagnosis of non-alcoholic fatty liver disease.⁷ And so it is critical to develop non-invasive diagnostic method to diagnose nonalcoholic fatty liver rather than invasive techniques. MicroRNAs (miRNAs) are small non-coding RNAs that have been discovered to play a vital role in transcriptional control mechanisms and guiding metabolic homeostasis.⁸ Mi-RNAs are detectable in almost all body fluids like serum, plasma, and urine. They are resistant to extreme conditions, such as low or high pH, extreme temperature, and RNAs activity.^{9,10} They have been suggested as diagnostic biomarkers for non-invasive assessment of the pathological state of their origin organ from peripheral blood.¹¹ MicroRNA-122 is involved in hepatocyte proliferation and maturation by stimulating the expression of some specific genes. In addition, it interacts with numerous target genes involved in lipid and cholesterol metabolism.¹² Mi-RNA-122 is implicated in NASH development, such as fatty acid synthase (FAS), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), sterol regulatory element-binding protein-1c (SREBP-1c), and sterol regulatory element-binding protein 2 (SREBP-2).¹³ The circulating levels of miR-122 correlate with alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in patients with NAFLD compared with the classic liver function markers.¹²

Neurotensin (NT) is an amino acid peptide secreted mainly by neuroendocrine cells in the small intestine.¹⁴ It plays an important role in regulating food ingestion and fat absorption.¹⁵ An acute increase in intestinal NT release occurs shortly after food ingestion.¹⁶ Many studies have described the role of NT in facilitating lipid digestion and fat absorption in the small intestine.^{17,18} Recently, Li et al reported reduced intestinal fat absorption, along with protection from obesity and NAFLD, in NT-deficient mice fed with a high-fat diet. Moreover, the authors demonstrated that higher plasma pro-NT levels in humans; were associated with insulin resistance and a higher risk of developing obesity later in life in non-obese individuals.¹⁹ There were no previous studies on serum proneurotensin level in NAFLD and so, to our knowledge, our current study is considered the first study on this issue. The aim of this study was to assess the expression of serum mi-RNA-122 and detection of serum proneurotensin status in a group of Egyptian patients with nonalcoholic fatty liver disease.

Patients and Methods

This case–control study was conducted on 157 patients with proven NAFLD Patients who were recruited from the outpatient clinic of the Faculty of Medicine, Minia University and Cairo Universities between October 2020 and September 2021. NAFLD diagnosis was proven by abdominal ultrasonography and laboratory investigations. Detailed history and complete laboratory examination were done for all participants including a patient history: name, age, history of smoking, drug history, and family history of any chronic diseases, complete clinical examination and anthropometric measurements that were performed to all patients and controls, the body mass index (BMI) was calculated as weight (in kilograms) divided by height squared (meters squared), liver function tests (alanine transaminase [ALT], aspartate transaminase [AST], gamma-glutamyltransferase [GGT], alkaline phosphatase [ALP], albumin), INR, CRP, Hb, total bilirubin, and total leukocytic count, lipid profiles (triglycerides [TG], high-density lipoprotein [HDL], low-density lipoprotein [LDL] cholesterol) and Alpha-feto protein [AFP].

Nonalcoholic fatty liver disease is characterized by excessive fat accumulation in the liver. It is associated with insulin resistance, central adiposity, increased BMI, hypertension, and hyperlipidemia, which collectively are features of metabolic syndrome. Diagnosis is usually made after an incidental discovery of unexplained elevation of liver enzyme levels or when steatosis is noted on imaging (eg, ultrasonography). Patients are often asymptomatic, and the physical examination is often unremarkable. Not a single laboratory test is diagnostic, but tests of liver function, tests for metabolic syndrome, and tests to exclude other causes of abnormal liver enzyme levels are routinely performed. Imaging studies, such as ultrasonography, computed tomography, and magnetic resonance imaging, can assess hepatic fat, measure liver and spleen size, and exclude other diseases.²⁰ We excluded patients with history of viral hepatitis (hepatitis B virus and hepatitis C virus), autoimmune hepatitis, hepatocellular carcinoma, alcohol consumption, liver cirrhosis, diabetes mellitus, Wilson's disease, secondary cause of hepatitis, drug-induced hepatitis, or obstructive biliary disease. One-hundred apparently healthy subjects with normal laboratory investigations and abdominal ultrasound were included as a control group.

A written informed consent was taken from all subjects, and the study was approved by the Research Ethics Committee. This study was performed in compliance with the ethics principles of the declaration of Helsinki for Good Clinical Practice guidelines.

Blood Sampling

Venous blood (10 mL) was drawn from participants after an 8–12 h fast. A 3-mL portion was collected into dry sterile vacutainer tubes and used for serum separation was left to clot at room temperature, and then centrifuged at $3000 \times g$ for 10 min at 4°C . Another 3-mL tube used for sera separation for the detection of micro 122 and pro-neurotensin was stored at -80°C until use. Liver function tests (alanine transaminase [ALT], aspartate transaminase [AST], gamma-glutamyltransferase [GGT], alkaline phosphatase [ALP], albumin), and lipid profiles (triglycerides [TG], and high-density lipoprotein [HDL] and low-density lipoprotein [LDL] cholesterol) creatinine were assayed in sera by Beckman automated biochemistry analyzer. The 2-mL tubes containing EDTA for complete blood picture by phoenix 3300 and 2 mL were collected in tubes containing sodium citrate for INR detection.

Detection of Serum Pro-Neurotensin

Serum pro-neurotensin was determined by enzyme linked immunosorbent assay (ELISA) using kits obtained from science and technology company (Bioassay Technology Laboratory SH. China), according to manufacturer's instructions the test was done.

Detection of Micro 122

Total RNA extraction and purification was done using a miRNeasy Mini Kit; cat no: 217004 (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized by reverse transcription reaction using TaqMan MicroRNA Reverse Transcription Kit; cat no: 4366596 (Applied Biosystems, Foster City, USA) and the thermal cycler (Quanta Biotech). The quantification of miR-122 level was amplified from cDNA using TaqMan universal Master Mix and TaqMan assay (has-miR-122; Catalog no: 4427975; Assay ID: 002245). The RNU49 was used as housekeeper gene (RNU49; Cat no: PN4427975; ID: 001005). All the samples were analyzed using the 5 plex Rotor-Gene PCR Analyzer (Qiagen, Germany). The $2^{-\Delta\Delta C_t}$ method was conducted for the analysis of gene expression levels using TaqMan microRNA Control Assays RNU49 as an endogenous reference control for normalization purposes.²⁰

Statistical Analysis

Data were entered and cleaned using Excel and exported to IBM SPSS software, version 21.0 for Statistical analysis. Descriptive statistics such as mean, median, SDs and percentage were used to describe the patients' characteristics and barriers to access to care. The relationships between patient characteristics and follow-up status were done by proper statistical test (the Chi-Square test and Fisher's exact test). All significant relationships with a p value <0.05 were considered for further analysis in binary logistics regression models. Of all statistical tests used, P-values less than 0.05 are considered significant.

Results

Our study included 157 patients with NAFLD (86 males and 71 females) with a median age of 56 years. Healthy controls were 100 subjects (57 males and 43 females) with a median age of 38 years. There was a statistically significant increase in the BMI, ALT, AST, ALP, GGT, total bilirubin, and serum albumin, INR, and AFP in NAFLD patients compared to the control group, while serum creatinine, hemoglobin and total leucocytic count showed non-significant differences between both groups (Table 1). NAFLD patients had a higher significant level of serum triglycerides, total cholesterol, HDL-cholesterol, and LDL-cholesterol compared to the controls (Table 1).

Table 2 and Figure 1 show that the median levels of serum proneurotensin and miRNA-122 were significantly higher in NAFLD patients compared to the control group, MI all the laboratory parameters and all histological findings. The level of miRNA-122 had a significant positive correlation with ALT, AST, total bilirubin, CRP, total leucocytic count, AFP, INR, ALP, and total cholesterol, whereas it showed negative correlation with HDL-cholesterol. In contrast, the level

Table I Demographic and Biochemical Parameters Between NAFLD Patients and Controls

	NAFLD Group (n=157) (Range, Median and IQR)	Control Group (n=100) (Range, Median and IQR)	P-value
Age (years)	28–80 56 (50–61)	19–77 38 (32–47)	<0.001
Gender (n, %)			
Male	86 (54.8%)	57 (57.0%)	0.727
Female	71 (46.2%)	43 (43.0%)	
BMI (kg/m ²)	22–41 30 (25–35)	20–35 25 (21–30)	<0.001
BMI (n, %)			
Average (20 - < 25)	17 (10.8%)	45 (45%)	<0.001
Overweight (25 - < 30)	46 (29.3%)	31 (31%)	
Obese (> 30)	94 (59.9%)	34 (34%)	
ALT (IU/L)	19–66 34 (28–44.5)	20–41 29 (26–36)	0.001
AST (IU/L)	24–125 45 (34–64)	24–58 35 (26–38)	<0.001
Total Bilirubin (mg/dl)	0.3–2.3 1.0 (0.8–1.3)	0.4–1.0 0.8 (0.65–0.9)	<0.001
Serum Albumin (g/L)	2.1–4.3 3.7 (3.3–3.9)	3.6–4.3 3.8 (3.6–4.0)	<0.001
INR	1.0–2.18 1.27 (1.19–1.43)	0.9–1.2 0.98 (0.95–1.0)	<0.001
ALP (IU/L)	12–468 86 (71–133.25)	20–41 29 (26–36)	0.001
GGT (IU/L)	15–105 57 (40–70)	24–59 36 (25.5–41)	<0.001
AFP (IU/L)	4–45 9 (7–13)	2.9–23 6 (5–7)	<0.001
Serum Creatinine (IU/L)	0.4–1.21 1.0 (0.8–1.1)	0.7–1.21 1.0 (0.8–1.1)	0.679
Hemoglobin (g/dl)	7.8–15.8 10.6 (10–12.3)	7.8–16.0 10.7 (10–12.65)	0.516
Total leucocytic count (x 1000/cmm)	2.7–29 7.1 (4.4–9.01)	2.0–29 6.35 (4.4–8.9)	0.920
C-reactive protein (mg/dl)	1.02–56 14.0 (10–25)	0.2–18 5.0 (4.0–6.5)	<0.001
Serum Triglycerides (mg/dl)	87–300 190 (170–200)	121–245 152 (134.5–165)	<0.001
Serum Cholesterol (mg/dl)	160–270 190 (180–202.5)	100–256 156 (150–186)	<0.001
Serum HDL (mg/dl)	20–60 32 (29–42.25)	22–60 42 (39.5–45)	<0.001
Serum LDL (mg/dl)	99–202 120 (102–150.5)	89–167 107.5 (102–125)	<0.001

Note: P value is significant if <0.05 and non-significant if >0.05.

Abbreviations: IQR, interquartile range; BMI, body mass index; ALT, alanine transaminase; AST, aspartate transaminase; INR, international normalized ratio; ALP, alkaline phosphatase; GGT, gamma-glutamyltransferase; AFP, alpha-feto protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table 2 Difference Between Groups Regarding Serum Proneurotensin and Micro-RNA-122 Assay

	NAFLD Group (n=157) (Range, Median and IQR)	Control Group (n=100) (Range, Median and IQR)	P-value
Serum Proneurotensin (pmol/L)	27–200 156 (138.3–177)	34–180 80 (70–98)	<0.001
Micro-RNA 122	1.0–90.51 8.0 (2.0–32.0)	1.0–64.0 2.0 (1.4–98)	<0.001

Note: P value is significant if <0.05 and non-significant if >0.05.

of serum proneurotensin showed positive correlation with hemoglobin and negative correlation with total leucocytic count (Table 3).

The ROC curve demonstrated that serum proneurotensin and mi-RNA-122 can differentiate between NAFLD patients and healthy controls at a cut-off ≥ 108 , ≥ 6.83 respectively. The AUC for serum proneurotensin was 0.811 with a sensitivity (80.0%) and specificity (80.0%) followed by mi-RNA-122 with AUC = 0.654 and a sensitivity (51.0%) and a specificity (70.0%) (Table 4, Figure 2).

Discussion

Liver biopsy is the gold standard invasive procedure that takes very small samples from different areas within the liver tissue to investigate the number of liver cells containing fat droplets (steatosis), the degree of liver cell damage, inflammation and the degree of liver fibrosis.²¹ It can help in the diagnosis of NAFLD severity by providing information about grading, staging, and prognosis. However, it is an invasive procedure with several limitations.²² Many non-invasive tests are available and may reduce the need for liver biopsy but are not specific to NAFLD, such as alpha-fetoprotein and many other nonspecific markers. AFP is a glycoprotein that is normally generated during conception by the fetal liver. In clinical practice, AFP levels are elevated in many clinical situations such as hepatocellular carcinoma, acute or chronic viral hepatitis, chronic liver disease, and gonadal tumors.^{23–39}

Mi-RNAs play an important role in many liver diseases such as viral hepatitis, cancer, and liver fibrosis.²⁴ MicroRNA-122 is the most abundant hepatic miRNA; it represents 70% of the total hepatic miRNAs.¹² A large number of studies are interested in the identification and validation of a simpler, non-invasive biomarker for the detection of NAFLD.

Our study showed a significant increase in the level of mi-RNA-122 in NAFLD patients compared to healthy controls. This comes in accordance with several studies who reported that mi-RNA-122 is significantly increased in

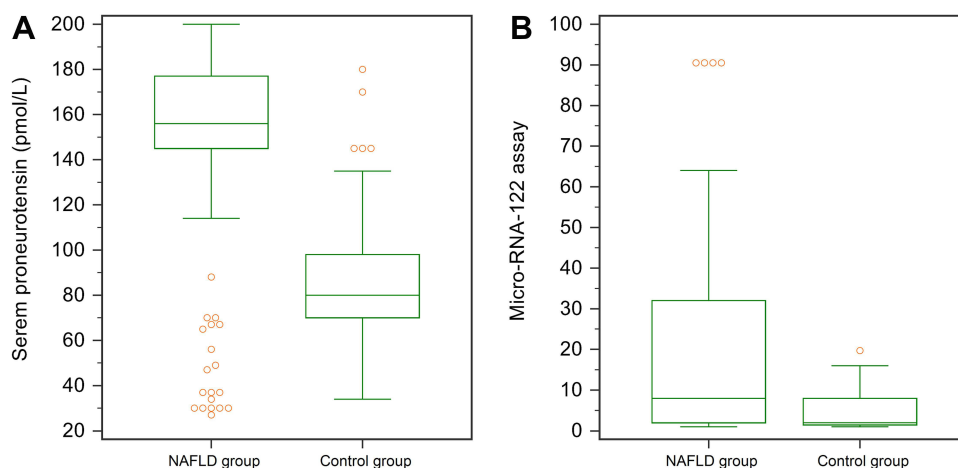


Figure 1 Box-and-whisker plot chart showing difference between groups regarding: (A) serum proneurotensin, (B) serum micro-RNA-122.

Table 3 Correlation Between Each of Serum Proneurotensin and Micro-RNA-122 and Other Measured Variables in NAFLD Group

NAFLD Group (n=157)	Micro-RNA 122	Proneurotensin
Age	0.059 (p=0.343)	0.015 (p=0.854)
BMI	0.012 (p=0.844)	0.019 (p=0.813)
Hb	0.016 (p=0.794)	0.196 (p=0.014)
Total leucocytic count	0.146 (p=0.019)	-0.186 (p=0.020)
CRP	0.150 (p=0.016)	-0.078 (p=0.332)
AFP	0.163 (p=0.009)	0.091 (p=0.261)
Albumin	-0.097 (p=0.119)	-0.052 (p=0.524)
INR	0.293 (p<0.001)	-0.032 (p=0.688)
ALP	0.138 (p=0.027)	0.046 (p=0.566)
GGT	0.058 (p=0.351)	0.184 (p=0.021)
Total Bilirubin	0.268 (p<0.001)	0.026 (p=0.751)
ALT	0.128 (p=0.040)	-0.041 (p=0.612)
AST	0.263 (p<0.001)	-0.053 (p=0.516)
TG	0.031 (p=0.620)	0.029 (p=0.722)
Serum Cholesterol	0.155 (p=0.013)	-0.006 (p=0.945)
HDL	-0.224 (p<0.001)	-0.160 (p=0.046)
LDL	-0.060 (p=0.341)	-0.131 (p=0.105)
Serum Creatinine	0.066 (p=0.295)	0.107 (p=0.186)

Note: Data presented as Spearman correlation coefficient (its p value).

Table 4 Cut off Value of Pro-Neurotensin and microRNA 122 in Diagnosis of NAFLD

	Cut-off	Sensitivity (%)	Specificity (%)	PPV	NPV
Serum Proneurotensin (pmol/L)	≥ 108	80.0%	80.0%	86.1%	72.2%
Micro-RNA 122	≥ 6.83	51.0%	70.0%	72.5%	47.9%

Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

the serum of NAFLD patients.^{22,26} Pirola et al reported that miR-122 levels are 7.2 times higher in patients with NASH compared to healthy subjects, suggesting it as an extrahepatic NASH marker.¹² On the contrary, Cheung et al found that mi-RNA-122 expression was significantly decreased in NASH patients. The authors confer its downregulation to the alteration of hepatic lipid metabolism genes.²⁷

We found that the levels of mi-RNA-122 were positively correlated with the serum levels of AST, ALT, and ALP. These findings were in agreement with Waidmann et al who found that there is a highly significant correlation between serum miR-122 levels and alanine aminotransferase (ALT), aspartate aminotransferase (AST),

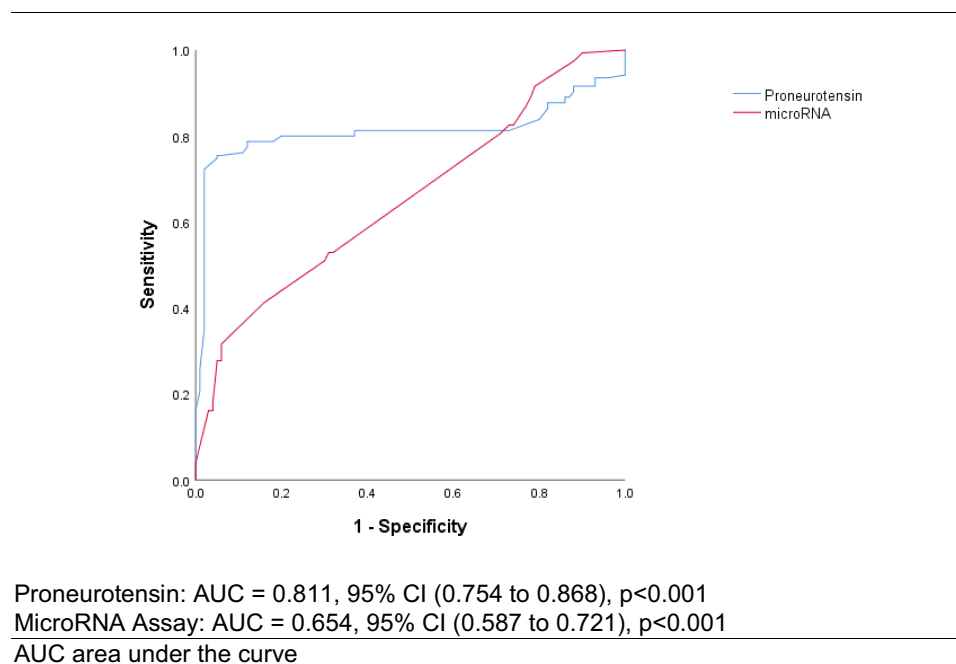


Figure 2 ROC curve for diagnosis of pro-neurotensin and microRNA 122 assay in NAFLD patients.

γ -glutamyltransferase (GGT) and alkaline phosphatase (ALP). Therefore, miRNA-122 may behave as a biomarker for hepatic injury.²⁸

Although we found that the serum miRNA-122 expression in NAFLD was significantly associated with high levels of total bilirubin, the international normalized ratio (INR), and C-reactive protein (CRP), Waidmann et al reported that miRNA-122 has a negative correlation with the international normalized ratio (INR), total bilirubin, and total protein levels.²⁸ Additionally, the current study showed that there is a positive relationship between over-expression of miRNA-122 and elevated serum of cholesterol, TG, and LDL. This is comparable to Gao et al, who found that the plasma levels of miR-122 was increased in patients with hyperlipidemia, and positively correlated with cholesterol, TG, and LDL-C levels, suggesting that the circulating miR-122 might be used as a target biomarker for hyperlipidemia.²⁹

The association between altered expression of serum NT and pathophysiology of NAFLD and whether there is a relation between susceptibility and NASH is largely unknown. Previous studies found an association between higher pro-NT and the development of obesity and cardiometabolic diseases.^{30,31} We observed that the circulating level of serum pro-neurotensin is significantly higher in patients with NAFLD. In addition, Barchetta et al observed a linear association between pro-NT, hepatic damage in NASH, and parameters related to glucose metabolism impairment. The authors assume that increased pro-NT levels help the absorption of fatty acids from the small intestine, leading to fat accumulation at specific sites, such as the liver.³²

Regarding the diagnostic value, the AUC for serum proneurotensin was 0.811 with a sensitivity (80.0%) and specificity (80.0%) followed by mi- RNA-122 with AUC = 0.654 and a sensitivity (51.0%) and a specificity (70.0%) ($p < 0.001$). Taking into account that a diagnostic tool is good if the AUROC is > 0.8 and excellent if the AUROC is > 0.9 ³³ therefore, the serum pro-NT may have a better diagnostic value and serve as a good predictor for NAFLD diagnosis compared to mi-RNA -122. The current study is the first to investigate the circulating level of serum pro-neurotensin in the patients with NAFLD, and the results showed that the median serum proneurotensin was significantly higher in group I [NAFLD Group] when compared to group II [Control Group]. Although the median micro-RNA-122 was significantly higher in group I, it was considered statistically lower when compared with the serum pro-neurotensin level elevation, suggesting that circulating pro-neurotensin might be used as a novel biomarker for patients with NAFLD.

Conclusion

In conclusion, we found that the levels of serum pro-NT may help as a non-invasive biomarker for NAFLD prediction and diagnosis. In addition, increased serum level of micro-RNA-122 may identify the presence of NAFLD. Large scale studies are warranted to investigate the possible role of mi-RNA-122 and pro-NT in the development, progression, and prognosis of NAFLD and NASH.

Human and Animal Rights

No animals were used in this research. All human research procedures were followed in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013.

Standards for Reporting

STROBE guidelines were followed for this case-control study.

Data Sharing Statement

Data are available upon reasonable request from the corresponding author.

Ethics Approval and Consent to Participate

The study was approved by the Research Ethics Committee of Minia University.

Consent for Publication

Informed consent was signed by every patient before enrolment in the study.

Funding

There is no funding to report.

Disclosure

The authors declare no conflict of interest, financial or otherwise.

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