https://doi.org/ 10.33472/AFJBS.6.Si3.2024.169-188

The Value of Serum Proneurotensin as A Predictor of Cardiovascular Risk in NAFLD Patients

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Article History
Volume 6,Issue Si3, 2024
Received:21 Mar 2024
Accepted : 08 May 202
doi: 10.33472/AFJBS.6.Si3.2024.169-188

Abstract

Background Cardiovascular disease (CVD) is the leading cause of death in NAFLD patients. Increased plasma proneurotensin (pro-NT) levels were found to identify the presence and severity of NAFLD. The association between pro-NT and incident major cardiovascular events has been confirmed. **Aims** To evaluate the association of serum pro-neurotensin with cardiovascular risk in NAFLD patients. **Methods** A cross-sectional cohort included sixty patients with NAFLD and documented CVD, sixty patients with NAFLD without documented CVD and 60 healthy controls. Liver function tests, liver enzymes, lipid profile, fasting blood glucose, abdominal ultrasound and serum proneurotensin were done. **Results** Proneurotensin was significantly higher in the NAFLD group as compared to controls, P <0.0001. Proneurotensin levels were significantly higher in patients with established CVD in addition to NAFLD as compared to NAFLD only patients P <0.0001. Serum proneurotensin showed significant positive correlation with Framingham score and NAFLD Fibrosis Score P <0.0001. Reciever operating characteristic (ROC) curve demonstrated a cut-off value >24.5 ng/L for proneurotensin as a predictor of NAFLD with 64.2% sensitivity and 96.7% specificity, (AUC) = 0.862, P < 0.001. And a cut-off value >28.5 ng/L for proneurotensin as a predictor of CVD in NAFLD patients, with 83.3% sensitivity and 78.3% specificity, (AUC) = 0.854, P < 0.001. **Conclusions** serum proneurotensin is significantly higher in NAFLD patients established CVD than NAFLD only patients and healthy subjects. It can be considered as a marker of CVD risk in such patients.

Introduction

The worldwide prevalence of NAFLD was estimated in a recent study, published in July 2022, to be 32.4%, higher in men (39.7%) than women (25.6%) [1].

Yet, the clinical importance of NAFLD is often underrated. An ultrasound diagnosis of fatty liver is usually a diagnosis of reassurance. Indeed, NAFLD ranges from non-alcoholic fatty liver that follows a non-progressive course. It also includes non-alcoholic steatohepatitis (NASH) that carries higher risks of fibrosis, cirrhosis with subsequent liver cell failure and hepatocellular carcinoma.[2]

Recently, cardiovascular disease (CVD) is considered to be the leading cause of death in NAFLD patients.[3,4]

Numerous epidemiological studies have reported an increased incidence of adverse CV events in NAFLD subjects compared with the general population.[5-9]

However, a clear mechanism for the causal impact of NAFLD on CVD that is independent from conventional metabolic risk factors is yet to be elucidated.

Neurotensin (NT), a 13–amino acid peptide mainly secreted by neuroendocrine cells in the small intestine[10], displays an important role in regulating food ingestion and fat absorption.[11] By doing so, NT influences energy balance and body weight[12]. NT mainly acts as a neurotransmitter in the central nervous system and as a hormone in the periphery, exerting its physiological action by binding the specific NT receptors.

Measurement of neurotensin is challenging because of its instability. Proneurotensin is a stable profragment of neurotensin that is considered to provide a surrogate to neurotensin levels.[13]

Increased plasma pro-NT levels was found to identify the presence and severity of NAFLD in 260 consecutive patients plus 60 selected as obese[14].

The association between pro-NT and incident major cardiovascular events has been confirmed in the Framingham Heart Study Offspring cohort, independently of the presence of traditional cardiovascular risk factors.[13]

Neurotensin binds to 2 receptors found in cardiovascular tissue: NTS1 and NTS2, with different effects. NTS1 has a higher affinity to neurotensin and directly stimulates cardiac function. The response includes increased heart rate and contractility with subsequent blood pressure elevation. Experimental dose-dependent effects of neurotensin was demonstrated on blood pressure, heart rate, myocardial contractility, vascular tone, permeability, and endothelial cell survival.[15-18]

The authors view these effects as potentially compensatory to metabolic dysfunction associated with CVD risk. This role, however, may have deleterious effects, as worsening hypertension, endothelial dysfunction and atherosclerosis[13].

This study aims to evaluate the association of serum pro-neurotensin with cardiovascular risk in NAFLD patients.

Population of study

This study is a Cross-sectional controlled cohort study**.** Patients with a clinical diagnosis of NAFLD were consecutively enrolled into the study until the desired sample size was reached. Patients with overt diabetes or body mass index (BMI) above 35 kg/ m² were excluded. One hundred and twenty Egyptian patients with NAFLD were enrolled. Sixty of which had no history of cardiovascular disease, including ischemic heart disease, chest pain or equivalent symptoms, cerebrovascular stroke and/or peripheral arterial disease. The other 60 had a documented history of atherosclerotic cardiovascular disease. Sixty age and gender matched healthy control subjects were included.

Ethical considerations:

The study protocol was approved by the Faculty ethical committee. A written informed consent was obtained from each patient or from their eligible surrogates.

Methods

Full history taking and clinical examination was done to all subjects. Blood pressure measurements was obtained according to Guidelines of the International Society of Hypertension as follow: Three blood pressure readings were obtained at 1-minute intervals, the second and third systolic and diastolic pressure readings were averaged and used in the analyses. Body weight was measured in light clothing and without shoes to the nearest 0.5 kg. Height was measured at the nearest 0.5 cm. BMI was calculated as weight (kg) divided by height (m^2). At ultrasounds, 4 parameters were tested[19]: (1) diffuse hyperechoic echotexture ("Bright liver"); (2) increased liver echotexture compared with the kidneys; (3) vascular blurring; (4) deep attenuation

The control subjects had no evidence of fatty liver at ultrasounds. They were matched with NAFLD cases for age and sex.

Blood samples were obtained from all subjects for lab assessment: Liver function tests, liver enzymes, lipid profile, fasting blood glucose

Serum proneurotensin measurement: Blood was collected from all subjects at the morning and after an overnight fast. Subjects were supine for approximately five to ten minutes prior to phlebotomy. Blood samples were immediately centrifuged, and plasma and serum was stored at -70° C. Concentrations of pro-NT was measured using a one step enzyme-linked chemiluminescence immunosorbent assay (SphingoTec, GMBH; Henningsdorf, Germany)[20].

NAFLD fibrosis score was calculated for all patients[21]. Framingham risk score was calculated for all patients.[22]

Results

The study comprised 120 NAFLD patients, 60 with established cardiovascular disease (CVD) and 60 without any history of cardiovascular events. Also 60 age and gender matched healthy control subjects (without NAFLD). Summary statistics of the 3 study groups, NAFLD only, NAFLD + CVD and controls, is available in Supplement 1, 2 and 3 respectively.

1. **Summary and comparison of categorical and variables among the 3 study groups:**

No significance difference in age and gender distribution among the different groups was noted. The frequency of smoking and hypertension also showed no significance difference. As regards Framingham risk category, patients with NAFLD without established CVD, had less frequency of "low" scores than controls (65% versus 91.7%), higher "intermediate" (30% vs. 8.3%) and "high" score frequencies (5% vs. 0%). P=0.0015. As regards NAFLD fibrosis score category, all NAFLD patients with established CVD fell into the "indeterminate" category, as compared to NAFLD only patients with 90% "indeterminate" and 10% "F0-F1" category, P=0.0123. **(Table 1)**

Table 1: Comparison among subjects of the 3 study groups regarding categorical variables

	Groups						P[a]
	Controls n=60		NAFLD only n=60		NAFLD + CVD n=60		
	N	%	N	%	n	%	
Gender							
Male	30	51.7	31	50.0	30	51.7	0.9780

Female	29	48.3	30	50.0	29	48.3	
Smoking	23	38.3	23	38.3	33	55.0	0.1048
HTN	-	-	27	45.0	36	60.0	0.1013[b]
Framingham Risk					--	--	0.0015
Category	55	91.7	39	65.0			
Low	5	8.3	18	30.0			
Intermediate	0	0	3	5.0			
High							
NAFLD fibrosis score	-	-					0.0123[b]
category			6	10.0	0	0	
F0-F1			54	90.0	60	100	
Indeterminate			0	0	0	0	
F3-F4							

[a]Chi-squared; [b]NAFLD only vs. NAFLD+CVD; [c]three groups

2. Comparison of all NAFLD patients versus controls:

SBP was significantly higher in the NAFLD group as compared to controls. FBS was significantly higher in the NAFLD group as compared to controls. Lipid profile was significantly worse in the NAFLD group, in the form of higher total cholesterol, LDL and triglycerides as well as lower HDL (as compared to controls). All liver enzymes were significantly higher in the NAFLD group as compared to controls, as well as total and direct bilirubin. Whereas the serum albumin was significantly lower and INR slightly higher(statistically significant). Hemoglobin was slightly lower (statistically significant), and platelets were also significantly lower in the NAFLD group as compared to controls. NAFLD Fibrosis Score was significantly higher in the NAFLD group as compared to controls, expectedly. Proneurotensin was significantly higher in the NAFLD group as compared to controls. P-values are demonstrated in **Table 2.**

Table 2: Comparison between all NAFLD patients with and without cardiovascular disease(CVD) versus control subjects regarding continuous variables

Variable	All NAFLD patients n=120		Controls n=60		P [a]
	Median	Average Rank	Median	Average Rank	
Age, y	50	95.5	48	80.5	0.0679
SBP, mm Hg	130	103.5167	120	64.4667	<0.0001
BMI, kg/m²	29.8	93.9125	29.5	83.6750	0.2139
FBS, mg/dl	10	100.3167	89	70.8667	0.0003
Cholesterol, mg/dl	213	110.3167	188	50.8667	<0.0001
HDL, mg/dl	53	83.2792	55.5	104.9417	0.0085
LDL, mg/dl	130	116.9042	99	37.6917	<0.0001
TG, mg/dl	169	117.4833	126.5	36.5333	<0.0001
ALT, U/L	59	119.0167	29	33.4667	<0.0001
AST, U/L	57	118.5750	29	34.3500	<0.0001
ALP, U/L	100	111.5375	68.5	48.4250	<0.0001

GGT, U/L	32	106.3167	27	58.8667	<0.0001
T.BIL, mg/dl	0.92	114.4458	0.6500	42.6083	<0.0001
D.Bil, mg/dl	0.3	115.0917	0.1500	41.3167	<0.0001
Albumin, g/dl	3.7	68.6875	4.5	134.1250	<0.0001
PC, %	92.5	86.9750	96	97.5500	0.1909
INR	1.1	97.2	1.0	77.1000	0.0112
HB, g/dl	13.2500	84.0542	13.7500	103.3917	0.0188
TLC, thousands/cmm	6.6	90.0167	7.2500	91.4667	0.8602
PLT, thousands/cmm	174	68.6708	275.5	134.1583	<0.0001
NAFLD Fibrosis Score	-0.804	116.4625	-2.5400	38.5750	<0.0001
Proneurotensin, ng/L	86.0	112.1917	20.5	47.1167	<0.0001

[a] Mann-Whitney test

3. Comparison of NAFLD only patients versus controls:

SBP was significantly higher in the NAFLD only patients without established CVD as compared to controls. FBS was significantly higher in the NAFLD only group as compared to controls, as well as BMI. Lipid profile was significantly worse in the NAFLD only group, in the form of higher total cholesterol, LDL and triglycerides (as compared to controls). However HDL showed no difference. All liver enzymes were significantly higher in the NAFLD only group as compared to controls, as well as total and direct bilirubin. Whereas the serum albumin and PC were significantly lower. Platelets were significantly lower in the NAFLD only group as compared to controls. Framingham score was significantly higher in the NAFLD only group as compared to controls. NAFLD Fibrosis Score was significantly higher in the NAFLD only group as compared to controls, expectedly. Proneurotensin was significantly higher in the NAFLD only group as compared to controls. P-values are demonstrated in **Table 3**.

Table 3: Comparison between patients with NAFLD without cardiovascular disease(CVD) versus control subjects regarding continuous variables

Variable	NAFLD only n=60		Controls n=60		P [a]
	Median	Average Rank	Median	Average Rank	
Age, y	50	65.5	48	55.5000	0.1146
SBP, mmHg	130	73.4917	120	47.5083	<0.0001
BMI, kg/m²	30.5	67.6083	29.5	53.3917	0.0252
FBS, mg/dl	101	69.3	89	51.7000	0.0056
Cholesterol, mg/dl	204.5	74.15	188	46.8500	<0.0001
HDL, mg/dl	55	59.4667	55.5	61.5333	0.7445
LDL, mg/dl	131.5	84.5667	99	36.4333	<0.0001
TG, mg/dl	166	86.5917	126.5	34.4083	<0.0001
ALT, U/L	51.5	87.5333	29	33.4667	<0.0001
AST, U/L	45	86.65	29	34.3500	<0.0001
ALP, U/L	87	74.7667	68.5	46.2333	<0.0001
GGT, U/L	33	74.6667	27	46.3333	<0.0001

T.BIL, mg/dl	0.9	81.0583	0.65	39.9417	<0.0001
D.Bil, mg/dl	0.3250	83.9917	0.15	37.0083	<0.0001
Albumin, g/dl	3.8	40.6167	4.5	80.3833	<0.0001
PC, %	96	46.6750	100	74.325	<0.0001
INR	1.0	52.2667	1.0	68.7333	0.0057
HB, g/dl	13.55	55.1417	13.7500	65.8583	0.0913
TLC, thousands/cmm	6.8	61.8917	7.2500	59.1083	0.6610
PLT, thousands/cmm	177.5	39.7583	275.5	81.2417	<0.0001
Framingham score	7.9	72.7	5.3	48.3000	0.0001
NAFLD Fibrosis Score	-0.9050	85.0833	-2.5400	35.9167	<0.0001
Proneurotensin, ng/L	24.2500	76.1917	20.5	44.8083	<0.0001

[a] Mann-Whitney test

4. **Comparison of NAFLD patients with established cardiovascular disease versus controls:**
SBP was significantly higher in the NAFLD group with CVD as compared to controls. FBS was significantly higher in the NAFLD with CVD group as compared to controls. Lipid profile was significantly worse in the NAFLD group with CVD, in the form of higher total cholesterol, LDL and triglycerides as well as lower HDL (as compared to controls). All liver enzymes were significantly higher in the NAFLD group with CVD as compared to controls, as well as total and direct bilirubin. Whereas the serum albumin and PC were significantly lower and INR slightly higher(statistically significant). Hemoglobin was slightly lower(statistically significant), and platelets were also significantly lower in the NAFLD group with CVD as compared to controls. NAFLD Fibrosis Score was significantly higher in the NAFLD group with CVD as compared to controls, expectedly. Proneurotensin was significantly higher in the NAFLD group with CVD as compared to controls. P-values are demonstrated in **Table 4**.

Table 4: Comparison between patients with NAFLD with cardiovascular disease(CVD) versus control subjects regarding continuous variables

Variable	**NAFLD + CVD n=60**		**Controls n=60**		**P [a]**
	Median	Average Rank	Median	Average Rank	
Age, y	50	65.5	48	55.5	0.1141
SBP, mmHg	125	73.5417	120	47.4583	<0.0001
BMI, kg/m²	29.55	60.2167	29.5	60.7833	0.9289
FBS, mg/dl	100.5	71.3333	89	49.6667	0.0006
Cholesterol, mg/dl	217.5	86.4833	188	34.5167	<0.0001
HDL, mg/dl	49	47.0917	55.5	73.9083	<0.0001
LDL, mg/dl	127.5	89.2417	99	31.7583	<0.0001
TG, mg/dl	172.5	88.375	126.5	32.6250	<0.0001
ALT, U/L	61.5	90.5	29	30.5000	<0.0001
AST, U/L	61	90.5	29	30.5000	<0.0001
ALP, U/L	111.5	88.3083	68.5	32.6917	<0.0001
GGT, U/L	32	77.9667	27	43.0333	<0.0001
T.BIL, mg/dl	1	87.8333	0.65	33.1667	<0.0001
D.Bil, mg/dl	0.3	86.1917	0.15	34.8083	<0.0001
Albumin, g/dl	3.7	36.7583	4.5	84.2417	<0.0001
PC, %	83	39.625	96	81.3750	<0.0001
INR	1.2	82.1333	1.0	38.8667	<0.0001

HB, g/dl	13.2	52.9667	13.75	68.0333	0.0176
TLC, thousands/cmm	6.4	58.1417	7.25	62.8583	0.4574
PLT, thousands/cmm	174	37.5833	275.5	83.4167	<0.0001
NAFLD Fibrosis Score	-0.7225	87.8417	-2.54	33.1583	<0.0001
Proneurotensin, ng/L	108.75	88.1917	20.5	32.8083	<0.0001

[a] Mann-Whitney test

5. Comparison of NAFLD patients with established cardiovascular disease versus NAFLD only patients:

Patients with established CVD in addition to NAFLD had significantly lower BMI and HDL, as well as significantly higher total cholesterol as compared to NAFLD only patients. Regarding liver function tests, liver enzymes were significantly higher as well as total bilirubin and coagulation profile, in patients with established CVD in addition to NAFLD as compared to NAFLD only patients. NAFLD Fibrosis Score was significantly higher in patients with established CVD in addition to NAFLD as compared to NAFLD only patients. Proneurotensin levels were significantly higher in patients with established CVD in addition to NAFLD as compared to NAFLD only patients. P-values are demonstrated in **Table 5.**

Table 5: Comparison between patients with NAFLD with and without cardiovascular disease(CVD), regarding continuous variables

Variable	**NAFLD only n=60**		**NAFLD + CVD n=60**		**P [a]**
	Median	Average Rank	Median	Average Rank	
Age, y	50	61.8	50	59.2	0.6811
SBP, mmHg	130	62.9333	125	58.0667	0.4364
BMI, kg/m²	30.5	68.3083	29.55	52.6917	0.0139
FBS, mg/dl	101	58.2750	100.5	62.7250	0.4833
Cholesterol, mg/dl	204.5	50.4750	217.5	70.5250	0.0016
HDL, mg/dl	55	70.2	49	50.8000	0.0022
LDL, mg/dl	131.5	62.3	127.5	58.7000	0.5706
TG, mg/dl	166	56.	172.5	65.	0.1563
ALT, U/L	51.5	44.975	61.5	76.0250	<0.0001
AST, U/L	45	37.2583	61.0	83.7417	<0.0001
ALP, U/L	87	39.7167	111.5	81.2833	<0.0001
GGT, U/L	33	61.9833	32.0	59.0167	0.6398
T.BIL, mg/dl	0.9	46.9583	1	74.0417	<0.0001
D.Bil, mg/dl	0.325	63.3667	0.3	57.6333	0.3623
Albumin, g/dl	3.8	66.5417	3.7	54.4583	0.0535
PC, %	100	88.9083	83	32.0917	<0.0001
INR	1	33.9167	1.2	87.0833	<0.0001
HB, g/dl	13.55	60.9667	13.2	60.0333	0.8831
TLC, thousands/cmm	6.8	64.1167	6.4	56.8833	0.2544
PLT, thousands/cmm	177.5	64.5	174	56.5000	0.2076
NAFLD Fibrosis Score	-0.9050	51.1083	-0.7225	69.8917	0.0031
Proneurotensin, ng/L	24.25	45.6583	108.7500	75.3417	<0.0001

[a] Mann-Whitney test

6. Correlation of Proneurotensin levels with various continuous variables by univariate regression.

Proneurotensin showed significant positive correlation with all potentially confounding variables, including age, SBP, BMI, FBS, lipid profile (with the exception of HDL).

Liver enzymes and INR showed significant positive correlation with serum proneurotensin while albumin and PC showed significant negative correlation.

Platelet counts and total leucocytic count showed significant negative correlation with serum proneurotensin.

Framingham score showed significant positive correlation with serum proneurotensin.**(Figure 1)**

NAFLD Fibrosis Score showed significant positive correlation with serum proneurotensin.**(Figure 2)**

P-values are demonstrated in **Table 6.**

Table 6: Correlation of Proneurotensin levels with various continuous variables

Variable	Coefficient	95% CI	P [a]
Age, y	4.3201	3.1824 to 5.4577	<0.0001
SBP, mmHg	1.6979	1.2466 to 2.1492	<0.0001
BMI, kg/m²	6.7444	4.8403 to 8.6485	<0.0001
FBS, mg/dl	2.1608	1.5836 to 2.7380	<0.0001
Cholesterol, mg/dl	1.0500	0.7772 to 1.3228	<0.0001
HDL, mg/dl	-5.8986	-13.9463 to 2.1491	0.1498
LDL, mg/dl	1.8388	1.3774 to 2.3001	<0.0001
TG, mg/dl	1.4647	1.1087 to 1.8206	<0.0001
ALT, U/L	4.7840	3.6991 to 5.8690	<0.0001
AST, U/L	5.0478	3.9197 to 6.1759	<0.0001
ALP, U/L	2.5134	1.9158 to 3.1109	<0.0001
GGT, U/L	7.1384	5.3580 to 8.9188	<0.0001
T.BIL, mg/dl	262.7791	199.8472 to 325.7110	<0.0001
D.Bil, mg/dl	792.3813	595.4510 to 989.3115	<0.0001
Albumin, g/dl	-213.5566	-323.9670 to -103.1463	0.0002
PC, %	-12.8072	-21.0754 to -4.5391	0.0026
INR	200.6581	147.8538 to 253.4624	<0.0001
HB, g/dl	30.7642	-14.0155 to 75.5440	0.1769
TLC, thousands/cmm	-42.7788	-76.8240 to -8.7336	0.0141
PLT, thousands/cmm	-1.6206	-2.4867 to -0.7546	0.0003
Framingham score	20.2180	15.9925 to 24.4435	<0.0001
NAFLD Fibrosis Score	117.0256	66.9887 to 167.0626	<0.0001

[a]univariate regression

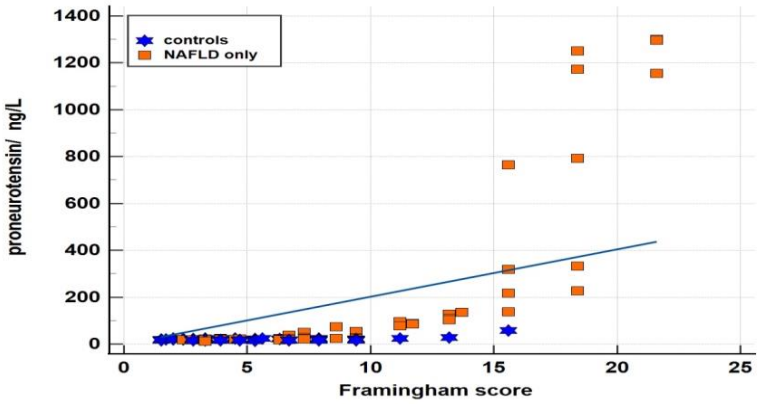

Figure 1: Scatter diagram showing correlation of proneurotensin levels with Framingham scores in patients without CVD and control subjects, n=120, Coefficient = 20.2180, 95% CI 15.9925 to 24.4435, P<0.0001

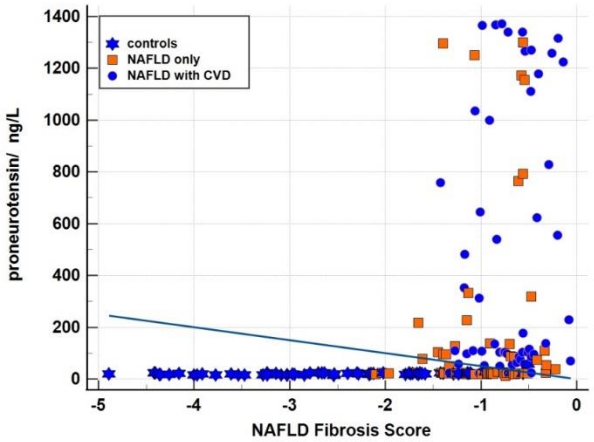

Figure 2: Scatter diagram showing correlation of proneurotensin levels with NAFLD fibrosis scores in the whole cohort, n=1b0, Coefficient = 117.0256, 95% CI : 66.9887 to 167.0626, P<0.0001

7. **Multiple comparison of proneurotensin among different study groups:**
Comparison of proneurotensin levels among the three groups shows a significant difference as follows: Control proneurotensin level values were significantly lower than each of the NAFLD only group and NAFLD+CVD group. The NAFLD only group proneurotensin levels were significantly lower than the NAFLD + CVD group but significantly higher than controls. Finally, the NAFLD + CVD group proneurotensin levels were significantly higher than each of the control group and the NAFLD only group. Thus showing a statistically significant "trend" among the 3 groups **(Table 7 and Figure 3).**

Table 7: Multiple comparison of proneurotensin(as a continuous variable) among different study groups

Groups	n	Median ng/L	IQR ng/L	Average Rank[a]	Kruskal-Wallis test	Jonckheere-Terpstra trend test
Controls	60	20.5	17.75 - 23	47.12		
NAFLD Only	60	24.25	21.5 - 107.25	91.35	P < 0.000001	P <0.00001
NAFLD With CVD	60	108.750	57.25 - 794.25	133.03		

[a]**Conover post hoc analysis**

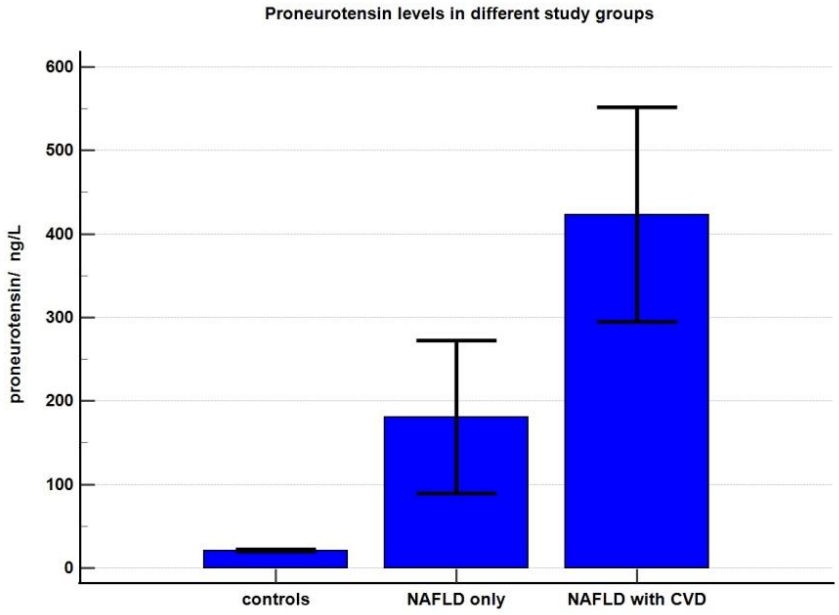

Figure 3: Proneurotensin levels in different study groups. Blue bars represent the mean values while the error bars in black represent 95% confidence intervals for the means.

8. **Multiple comparison of proneurotensin among different Framingham risk categories:** Comparison of proneurotensin levels as a continuous variable among the Framingham risk categories shows a significant difference as follows: "Low" risk patients had proneurotensin values that were significantly lower than each of the "intermediate" and "high" risk groups. There was NO difference, however between "intermediate" versus "high" risk groups as regards proneurotensin levels **(Table 8 and Figure 4).**

Table 8: Multiple comparison of proneurotensin(as a continuous variable) among different Framingham risk categories

Framingham risk categories	N 120	Median	IQR	Average Rank[a]	Significance	Kruskal-Wallis test	Jonckheere-Terpstra trend test
Low	94	21	18.0-23.5	48.06	vs. Intermediate and High		
Intermediate	23	110	80.125-296	103.78	vs. Low	P < 0.000001	P <0.00001
High	3	1296	1190.25 - 1299.75	118.33	vs. Low		

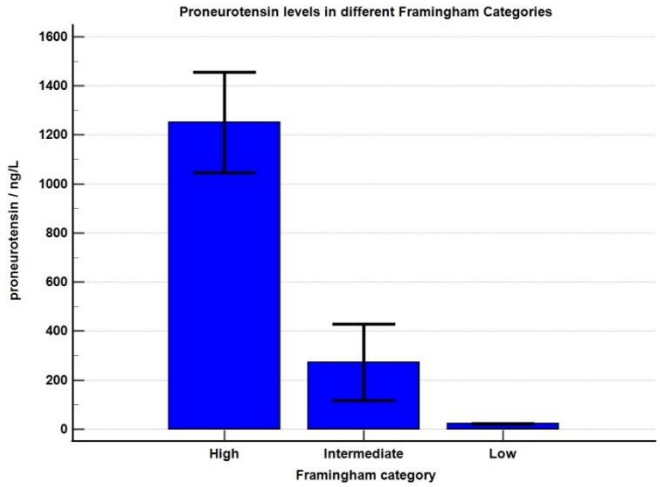

Figure 4: Comparison of Proneurotensin levels among different Framingham risk categories. Blue bars represent the mean and black error bars represent 95% confidence intervals for the mean. Proneurotensin levels were significantly higher in subjects with "high" Framingham risk versus the "low" risk group. It was significantly higher in the "intermediate" group than the "low" risk group. P value was <0.000001 by Kruskal Wallis test.

9. Reciever operating characteristic (ROC) curve analysis:

Reciever operating characteristic (ROC) curve was applied to test serum proneurotensin as a potential predictor of each of NAFLD and CVD in NAFLD patients.

At a cut-off value >24.5 ng/L, Proneurotensin can predict NAFLD with 64.2% sensitivity and 96.7% specificity. The area under the curve (AUC) = 0.862, P < 0.001. **(Figure 5)**

At a cut-off value >28.5 ng/L, Proneurotensin can predict CVD in NAFLD patients, with 83.3% sensitivity and 78.3% specificity. The area under the curve (AUC) = 0.854, P < 0.001. **(Figure 6)**

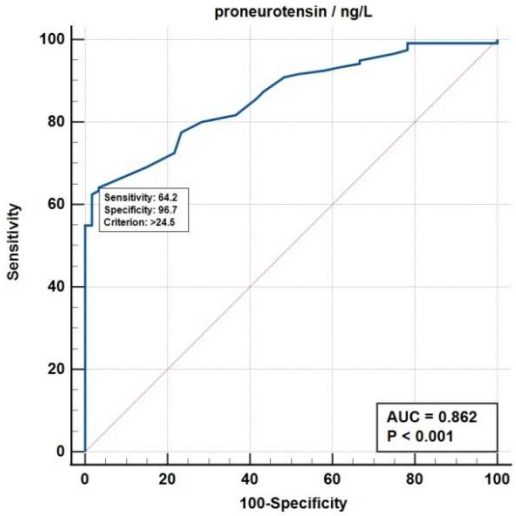

Figure 5: Reciever operating characteristic (ROC) curve testing Proneurotensin as a predictor of NAFLD. At a cut-off value >24.5ng/L, Proneurotensin can predict NAFLD with 64.2% sensitivity and 96.7% specificity. The area under the curve (AUC) = 0.862, P < 0.001.

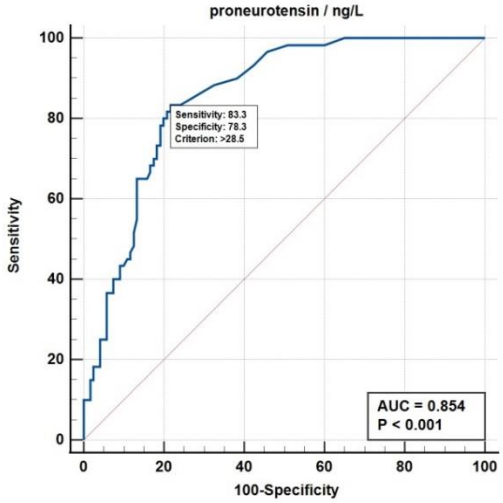

Figure 6: Reciever operating characteristic (ROC) curve testing Proneurotensin as a predictor of CVD in NAFLD patients. At a cut-off value >28.5ng/L, Proneurotensin can predict CVD in NAFLD patients with 83.3% sensitivity and 78.3% specificity. The area under the curve (AUC) = 0.854, P < 0.001.

Discussion

Proneurotensin levels were significantly higher in NAFLD patients in general as compared to control subjects. It was also significantly higher in NAFLD only patients (without established CVD) than control subjects. This data provides further validation for proneurotensin as a novel biomarker for NAFLD and metabolic dysregulation.[13,14,23-25]

In this study, liver enzymes and total bilirubin were significantly higher in patients with NAFLD and established CVD as compared to NAFLD only patients. Similarly, patients with NAFLD and established CVD had a worse coagulation profile. This shows a higher prevalence of CVD among NAFLD patients with a more severe disease in the form of worsening of liver function tests denoting steatohepatitis (NASH). This association has been demonstrated in previous literature.[26-28]
NAFLD Fibrosis Score was significantly higher in patients with NAFLD and established CVD as compared to NAFLD only patients. CVD risk was also demonstrated to be higher with progression of fibrosis in previous literature.[29]
In this study, proneurotensin levels correlated positively with Framingham risk scores in control patients and NAFLD patients. So, it is evident that elevated proneurotensin is associated with a higher risk for CVD. Januzzi et al have demonstrated a significant increase in incident CVD events with higher proneurotensin concentrations in 2016[13].
Yet, little is known concerning the nature of this association. Neurotensin is a locally acting hormone that has a wide range of physiological effects on the heart. It has got a regulatory role concerning contractility, blood pressure and the heart rate[30].
Increased levels of proneurotensin, therefore, may be viewed as a compensatory mechanism that leads to cardiac stimulation. This may have deleterious effects on the heart, probably through increased atherogenesis and cardiac load.
In this study, the association of elevated proneurotensin with CVD risk is further validated in the subset of patients with NAFLD. It is already known that NAFLD increases CVD risk via multiple mechanisms, some of which are independent from metabolic dysfunction. In fact, a study in 2018 stated that the impact of NAFLD as a risk factor for CVD was highest in patients without metabolic dysfunction.[31]
In this study, proneurotensin levels are significantly higher in NAFLD patients with established CVD than those without, given that NAFLD patients, in turn, already have got higher proneurotensin levels that control subjects. This points again at the suggested compensatory role for neurotensin even after cardiac injury, that may have a role in disease progression. This data suggests neurotensin, and its receptors (NTSR1 and NTSR2) as potential targets for primary and secondary prevention of CVD.
Interestingly, in this study, NAFLD Fibrosis Score showed significant positive correlation with serum proneurotensin. NAFLD severity was described as posing a higher risk of CVD development[32].
At a cut-off value >24.5ng/L, Proneurotensin could predict NAFLD with 64.2% sensitivity and 96.7% specificity. The area under the curve (AUC) = 0.862, $P < 0.001$. In a recent study, NAFLD was predicted at a cut off value >107 pmol/L with 84% sensitivity and 75% specificity, area under the curve was 0.836[14].
Another study demonstrated that proneurotensin had 80.0% sensitivity and 80.0% specificity at a cutoff ≥108pmol/L (area under the curve 0.811)[24].
At a cut-off value >28.5ng/L, Proneurotensin can predict CVD in NAFLD patients, with 83.3% sensitivity and 78.3% specificity. The area under the curve (AUC) = 0.854, $P < 0.001$. To date, no similar analysis was previously published in the literature as far as the authors know.

The study concludes that serum proneurotensin is significantly higher in NAFLD patients in general than healthy subjects. It is significantly higher in the subset of NAFLD patients with established CVD. In NAFLD patients without CVD, we found that serum proneurotensin is positively correlating with Framingham risk scores and is higher in those falling into high risk category. Thus,

it can prove to be an additional marker of cardiovascular risk. It can help in risk stratification of NAFLD patients to undertake an individualized approach of management.

Also, serum proneurotensin can predict NAFLD with excellent specificity but modest sensitivity. It can predict CVD in NAFLD patients with good sensitivity and moderate specificity. As a relatively new marker, further research may be required to

validate the cut-off value that best correlates with clinical data, especially NAFLD and cardiovascular disease.

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Supplemental Material

Table 1 showing summary statistics of the the NAFLD only group regarding continuous variables

	NAFLD only n=60					
	Min	**Max**	**Median**	**IQR**	**Mean**	**SD**
Age	41	59	50	45.5 - 55	50.1	5.0914
Systolic BP	100	150	130	120 - 135	128.083	10.4594
BMI	26.3	34.8	30.5	29.150 - 32.4	30.735	2.1819
Fasting Plasma Glucose	71	123	101	87.5 - 107	98.867	13.2530
Cholesterol	155	273	204.5	190 - 222.5	208.1	26.5136
HDL	30	72	55	48 - 61.5	54.667	8.5542
LDL	80	180	131.5	116 - 141.5	130.367	22.4424
TG	113	200	166	150 - 177	164.567	18.6687
ALT	29	77	51.5	40 - 61.5	51	12.7771
AST	30	70	45	38.5 - 52.5	46.083	10.0529
ALP	48	121	87	74.5 - 101	86.217	19.3120
GGT	16	53	33	27 - 39	33.2	7.9272
T.BIL	0.5	1.2	0.9	0.790 - 1	0.872	0.1587
D.Bil	0.1	0.55	0.325	0.210 - 0.4	0.317	0.1140
ALB	3.5	5	3.8	3.65 - 4	3.863	0.3031
PC	87	100	100	95.5 - 1	98.150	3.0963
INR	0.850	1.1	1	0.9 - 1.1	0.995	0.07849
HB	10.7	16.7	13.55	12.45 - 14.75	13.552	1.55
TLC	4	10.6	6.8	5.7 - 8.35	7. 7	1.6594
PLT	151	287	177.5	166.5 - 198	185	28.4557

Framingham Risk Score	2.4	21.6	7.9	6.3 - 13.2	9.537	5.2995
NAFLD Fibrosis Score	-2.1	0.221	-0.905	-1.272 - 0.629	-0.970	0.4379
Proneurotensin ng/L	12.5	1301	24.25	21.5 - 107.250	180.883	352.9673

Table 2 showing summary statistics of NAFLD + CVD group regarding continuous variables

	NAFLD + CVD n=60					
	Min	**Max**	**Median**	**IQR**	**Mean**	**SD**
Age	40	59	50	48 - 52.5	49.633	4.3917
Systolic BP	115	140	125	120 - 130	126.833	7. 89
BMI	25.4	34.7	29.550	27.9 - 31.250	29.680	2.3111
Fasting Plasma Glucose	77	123	1 .5	89 - 110.5	1 .817	12.9320
Cholesterol	195	243	217.5	208 - 227	217.917	12.2741
HDL	39	63	49	46 - 56	50.517	5.9788
LDL	101	160	127.5	123 - 134	128.517	10.4857
TG	136	201	172.5	156 - 182	169.2	17.0322
ALT	49	78	61.5	59 - 64	61.733	5.6624
AST	48	73	61	58 - 64.5	60.783	5.3523
ALP	86	133	111.5	99.5 - 122	110.850	13.6218
GGT	22	39	32	29 - 35	31.967	3.7 5
T.BIL	0.6	1.4	1	0.9 - 1.1	1.018	0.1690
D.Bil	0.15	0.5	0.3	0.25 - 0.35	0.305	0.09686
ALB	3.5	4.1	3.7	3.6 - 3.8	3.745	0.1501
PC	81	100	83	82 - 92	86.183	4.9386

INR	1	1.2	1.2	1.1 - 1.2	1.160	0.05272
HB	11.6	16.4	13.2	12.55 - 14.45	13.520	1.2352
TLC	4.4	10.5	6.4	5.55 - 8.05	6.722	1.6907
PLT	151	213	174	162 - 192	176.967	17.6913
NAFLD Fibrosis Score	-1.43	0.063	-0.722	-0.976 - 0.487	-0.721	0.3239
Proneurotensin ng/L	21	1371.5	108.750	57.25 - 794.25	423.283	497.778

Table 3 showing summary statistics of the control group regarding continuous variables

	Controls n=60					
	Min	**Max**	**Median**	**IQR**	**Mean**	**SD**
Age	42	57	48	46 - 52	48.667	4. 71
Systolic BP	105	130	120	115 - 125	120.583	7.1953
BMI	25.8	34.2	29.5	27.550 - 31.750	29.757	2.4665
Fasting Plasma Glucose	70	120	89	79 - 1	92	14.5381
Cholesterol	168	221	188	179 - 2	189.983	13.2223
HDL	45	67	55.5	51.5 - 58.5	55.533	5.3535
LDL	78	127	99	90 - 105	98.650	9.8477
TG	100	150	126.5	115.5 - 139	126.667	14.9334
ALT	21	44	29	26 - 32	29.783	4.7696
AST	21	44	29	26.5 - 33	30.317	4.9145
ALP	31	105	68.5	56.5 - 83	69.6	18.4788

GGT	16	36	27	24 - 31	26.817	4.8450
T.BIL	0.4	1	0.650	0.575 - 0.750	0.661	0.1334
D.Bil	0.05	0.3	0.15	0.1 - 0.2	0.148	0.05556
ALB	3.5	5.4	4.5	4 - 5	4.502	0.5522
PC	82	100	96	90 - 1	94.233	5.3784
INR	0.9	1.2	1	1 - 1.1	1.041	0.08361
HB	12.4	16.2	13.75	13.250 - 14.650	13.923	0.9446
TLC	4.3	10.8	7.25	5.65 - 8.05	6.902	1.6319
PLT	156	400	275.5	196.5 - 343	271.133	75.3967
Framingham Risk Score	1.5	15.6	5.3	3.3 - 7.9	5.912	3.2309
NAFLD Fibrosis Score	- 4.89	0.505	-2.54	-3.26 - 1.665	-2.563	1.1083
Proneurotensin ng/L	14.5	58.5	20.5	17.750 - 23	20.933	5.9357