JIM Original Article

doi: 10.1111/joim.13651

Higher circulating levels of proneurotensin are associated with increased risk of incident NAFLD

Francesca De Vito[1], Velia Cassano[1], Elettra Mancuso[1], Elena Succurro[1], Marta Letizia Hribal[1], Angela Sciacqua[1], Francesco Andreozzi[1], Giorgio Sesti[2] & Teresa Vanessa Fiorentino[1]

From the [1]Department of Medical and Surgical Sciences, University Magna Graecia of Catanzaro, Catanzaro, Italy; [2]Department of Clinical and Molecular Medicine, University of Rome-Sapienza, Rome, Italy

Abstract. De Vito F, Cassano V, Mancuso E, Succurro E, Hribal ML, Sciacqua A, et al. Higher circulating levels of proneurotensin are associated with increased risk of incident NAFLD. *J Intern Med.* 2023;**OO**:1–11.

Background. Neurotensin (NT), an intestinal peptide able to promote fat absorption, is implicated in the pathogenesis of obesity. Increased levels of proneurotensin (pro-NT), a stable NT precursor fragment, have been found in subjects with nonalcoholic fatty liver disease (NAFLD); however, whether higher pro-NT levels are associated with an increased NAFLD risk independently of other metabolic risk factors is unsettled.

Methods. Ultrasound-defined presence of NAFLD was assessed on 303 subjects stratified into tertiles according to fasting pro-NT levels. The longitudinal association between pro-NT levels and NAFLD was explored on the study participants without NAFLD at baseline reexamined after 5 years of follow-up ($n = 124$).

Results. Individuals with higher pro-NT levels exhibited increased adiposity, a worse lipid profile, and insulin sensitivity as compared to the lowest tertile of pro-NT. Prevalence of NAFLD was progressively increased in the intermediate and highest pro-NT tertile as compared to the lowest tertile. In a logistic regression analysis adjusted for several confounders, individuals with higher pro-NT levels displayed a raised risk of having NAFLD (OR = 3.43, 95%CI = 1.48–7.97, $p = 0.004$) than those in the lowest pro-NT tertile. Within the study cohort without NAFLD at baseline, subjects with newly diagnosed NAFLD at follow-up exhibited higher baseline pro-NT levels than those without incident NAFLD. In a cox hazard regression analysis model adjusted for anthropometric and metabolic parameters collected at baseline and follow-up visit, higher baseline pro-NT levels were associated with an increased risk of incident NAFLD (HR = 1.52, 95%CI = 1.017–2.282, $p = 0.04$).

Conclusion. Higher pro-NT levels are a predictor of NAFLD independent of other metabolic risk factors.

Keywords: gut hormone, liver damage, NAFLD, neurotensin, obesity, proneurotensin

Introduction

In the last decades, nonalcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease worldwide, representing a major health problem of global concern due to its associated hepatic and extrahepatic complications [1–6]. Given this clinical magnitude and the lack of effective treatments able to counteract progression of liver damage, it has become a priority identifying novel pathogenic and therapeutic targets.

Neurotensin (NT) is a 13 amino acid peptide released from neuroendocrine cells of the small intestine in response to a fat meal, which is able to promote fat digestion and absorption and regulate energy homeostasis [7–11]. Increasing evidence suggests that NT is involved in the pathogenesis of obesity and its related metabolic disorders [11–13]. NT exerts a wide range of physiological actions by binding three NT receptor (NTR) types: the two G-protein coupled NTR-1 and NTR-2, and the

non-G protein coupled NTR-3, known as sortilin, a member of the Vps10p-domain receptor family [10, 14]. The lack of NT in mice has been found to counteract intestinal fat absorption, high-fat diet-induced weight gain, hepatic steatosis, and glucose homeostasis perturbations [12]. Likewise, NTR-3 downregulation resulted in a reduced adipose tissue expansion, hepatic lipid accumulation, and improved insulin sensitivity in animal models of diet-induced obesity [13, 15]. In line with these preclinical evidence, indicating a pivotal role of NT in the development of obesity and its related metabolic disarrangements, higher circulating levels of proneurotensin (pro-NT), a stable NT precursor fragment produced in equimolar amounts relative to NT [16], have been found to be cross-sectionally linked to obesity, insulin resistance and type 2 diabetes (T2DM), and to predict incident obesity, T2DM, and adverse cardiovascular events [17–21].

Interestingly, several experimental studies have provided evidence that NT, in addition to regulate gut lipid influx and consequently adipose tissue expansion/dysfunction and hepatic fat deposition, may directly activate immune-inflammatory responses and anabolic/promitotic pathways such as mTOR and MAPK signaling pathways [22–27]. These pathways are known to be implicated in NAFLD pathogenesis [28–30], pointing toward a direct pathogenic role of an upregulated NT/NTRs axis in the development of NAFLD and its progression toward more severe forms of liver disease. Remarkably, morbidly obese subjects with biopsy-proven NAFLD exhibit higher levels of pro-NT, which correlates with the severity of hepatic steatosis, inflammation, and fibrosis independently of adiposity measures [31–33]. However, given the close relationship between NAFLD and altered glucose tolerance conditions, including prediabetes and T2DM [4, 34], whether higher levels of pro-NT are associated with NAFLD independently of glucose dysglycemic conditions remains to be firmly confirmed. Additionally, whether higher levels of pro-NT may confer an increased risk to develop NAFLD has not been explored yet. To address these questions, in this study, we first evaluated the association increased levels of pro-NT with NAFLD amongst nondiabetic subjects. Second, we sought to explore whether higher levels of pro-NT are an independent predictor of future development of NAFLD.

Methods

The study population encompasses 303 nondiabetic individuals participating in the CATAnzaro MEtabolic RIsk factors (CATAMERI) study, an ongoing longitudinal observational study enrolling White subjects at risk for metabolic and cardiovascular outcomes, whose design has been previously described in detail [4, 35, 36]. All individuals were recruited at the Department of Medical and Surgical Sciences of the University "Magna Graecia" of Catanzaro. Exclusion criteria were as follows: Diabetes mellitus defined according to the current ADA criteria, history of malignant or autoimmune diseases, heart or renal failure, acute infections or positivity for antibodies to hepatitis C virus or hepatitis B surface antigen, accumulation diseases such as amyloidosis and hemochromatosis, history of drug abuse, self-reporting alcohol consumption of >20 g/day, and history of treatments known to induce liver injury or affect glucose metabolism, including tamoxifen, glucocorticoids, tetracycline, estrogens, methotrexate, and amiodarone. The protocol of the study received the approval by the Hospital ethical committee (Comitato Etico Azienda Ospedaliera "Mater Domini"), and each study subject gave his/her written informed consent before to be enrolled in this investigation in accordance with principles of the Declaration of Helsinki.

Each study participant underwent a complete medical history, measurement of body mass index (BMI) and waist circumference, and assessment of body composition by bioelectrical impedance. After an overnight fasting, a biochemical characterization, including a 75 g OGTT, was performed in all study subjects. In accordance with the ADA criteria [37], individuals were categorized as having normal glucose tolerance when fasting plasma glucose was <100 mg/dL and 2-h post-load glucose <140 mg/dL, isolated impaired fasting glucose when fasting plasma glucose was 100–125 mg/dL and 2-h post-load glucose <140 mg/dL, and impaired glucose tolerance (IGT) when fasting plasma glucose was <100 mg/dL, and 2-h post-load glucose was 140–199 mg/dL.

Hepatic ultrasonography

A hepatic ultrasonography was carried out in all participants by the same trained operator, who was blind to their clinical data, using a Toshiba Aplio 50 ultrasound apparatus with a 3.5-MHz linear

transducer [4, 36]. Diagnosis of NAFLD was established on the basis of the following criteria: echo discrepancy between liver and kidney, augmented hepatic echogenicity, decreased echo penetration into the deep portion of the liver, and vascular blurring.

Longitudinal analysis

We excluded from this analysis subjects having NAFLD at baseline. Amongst subjects without ultrasound signs of NAFLD at baseline ($n = 167$), 124 individuals were reexamined after a mean follow-up of 5.1 ± 1.2 years, whereas 43 subjects were lost to follow-up. Anthropometric, biochemical characterization including OGTT and hepatic ultrasound were repeated at follow-up visit. Subjects were subdivided into two subgroups according to the presence or absence of newly ultrasound diagnosed NAFLD. Glucose tolerance at follow-up visit was defined according to ADA criteria [37].

Circulating pro-neurotensin assay

Circulating levels of pro-NT were measured in fasting serum samples, which were collected at baseline, immediately frozen after separation and stored at −80°C until use. Determination of serum Pro-NT concentrations was performed using an enzyme-linked immunosorbent assay to detect pro-NT amino acids 1 to 117 (MyBioSource, San Diego, CA, USA) following the manufacturers' instructions. Detection of luminescent signals was carried out using a plate reader luminometer with 450 nm filter (Varioskan LUX Multimode Microplate Reader, Thermo Fisher Scientific, Waltham, MA, USA). The analytical assay sensitivity was less than 4.78 pg/mL. Intra- and inter-assay coefficients of variability were less than 10% and 12%, respectively.

Biochemical parameters

Levels of glucose, triglycerides, total and HDL cholesterol levels were determined by enzymatic methods (Roche, Basel, Switzerland). Serum insulin concentrations were measured to be a chemiluminescence-based assay (Immulite, Siemens, Italy). Alanine aminotransferase and aspartate aminotransferase (AST) levels were measured using the α-ketoglutarate reaction, and gamma-glutamyltransferase (GGT) concentrations were determined with the L-gamma-glutamyl-3-carboxy-4-nitroanilide rate method (Roche, Basel, Switzerland). An automated instrument (CardioPhase hsCRP, Milan, Italy) was used to measure serum high sensitivity C reactive protein (hsCRP) concentrations.

Calculations

Different surrogate measures of insulin sensitivity were computed. The Matsuda index was calculated as 10,000/square root of [fasting glucose × fasting insulin] × [mean glucose × mean insulin during OGTT] [38]. The liver insulin resistance (liver IR) index was calculated using the following formula: −0.091 + (log insulin area under the curve [AUC] 0–120 min × 0.400) + (log fat mass % × 0.346) − (log HDL Cholesterol × 0.408) + (log BMI × 0.435) [39].

The severity of hepatic fibrosis was evaluated with the validated Fibrosis-4 (FIB-4) index [40]. We calculated FIB-4 as: (age [years] × AST [UI/L])/(platelet count [10^9/L] × ALT$^{1/2}$ [UI/L]) [40].

Statistical analyses

Statistical analyses were carried out by using SPSS software program Version 22.0 for Windows. Variables showing a skewed distribution such as pro-NT, triglycerides, fasting, and 2-h post-load insulin, AST, ALT, and GGT concentrations were natural log transformed for statistical analyses. Continuous variables are expressed as means ± SD. Categorical variables were compared by using a χ^2 test. A general linear model with post hoc Fisher's least significant difference correction for pairwise comparisons was employed to examine differences in clinical parameters among the study groups. A multivariate logistic regression analysis was used to determine the association between the pro-NT tertiles and presence of NAFLD. We included in the logistic regression analysis model the variables found to be significantly different among the three tertiles of pro-NT (BMI, total and HDL cholesterol, triglycerides, hsCRP, fasting, and 2-h post-load glucose and insulin levels) in addition to age and gender in order to evaluate whether the association between pro-NT levels and NAFLD is independent of potential confounders. We estimated the association between each SD increment of log-transformed pro-NT levels at baseline and hazard ratio (HR) to develop NAFLD by performing a Cox proportional hazard regression analysis. A

Table 1. *Clinical data of study subjects stratified into tertiles according to proneurotensin (pro-NT) levels*

	1 Tertile ($n = 100$)	2 Tertile ($n = 103$)	3 Tertile ($n = 100$)	p
Gender (M/F)	(44/56)	(44/59)	(47/53)	0.82
Age (years)	45 ± 13	48 ± 11	44 ± 13	0.06
BMI (kg/m^2)	30.5 ± 5.2	31.0 ± 7.6	32.2 ± 7.2*	0.05
Waist circumference (cm)	101 ± 14	103 ± 15	106 ± 16**	0.03
Fat mass (%)	33.7 ± 7.2	35.4 ± 8.8	37.2 ± 8.7	0.04
Total cholesterol (mg/dL)	198 ± 37	202 ± 39	197 ± 45	0.99
HDL cholesterol (mg/dL)	52 ± 15	51 ± 11	48 ± 10*	0.07
Triglycerides (mg/dL)	108 ± 44	127 ± 61*	129 ± 78*	0.04
Fasting glucose (mg/dL)	91 ± 10	93 ± 11	91 ± 10	0.75
2-h post-load glucose (mg/dL)	117 ± 25	119 ± 33	121 ± 29	0.79
Fasting insulin (μU/mL)	13 ± 7	14 ± 7	16 ± 10	0.40
2-h post-load insulin (μU/mL)	82 ± 56	85 ± 61	115 ± 68*	0.05
NGT/IFG/IGT/IFG-IGT	73/10/9/8	68/15/9/11	69/12/13/6	0.77
hsCRP (mg/L)	3.33 ± 4.32	3.74 ± 4.27	5.62 ± 6.31*#	0.04
AST (Ul/L)	22 ± 9	21 ± 7	23 ± 14	0.50
ALT (Ul/L)	23 ± 12	24 ± 14	28 ± 16*	0.08
GGT (Ul/L)	23 ± 16	23 ± 15	30 ± 30*	0.07
Pro-NT (pg/mL)	12 ± 9	105 ± 42***	318 ± 71***###	<0.0001
Matsuda index	2.9 ± 2.1	2.3 ± 1.5	1.1 ± 0.9***##	0.001
Liver IR index	2.5 ± 0.4	2.6 ± 0.4	2.7 ± 0.4**	0.02[a]
Hepatic steatosis *No* (%)	34 (34%)	41 (40%)*	61 (61%)**#	<0.0001
FIB-4 index	0.75 ± 0.3	0.86 ± 0.4	0.95 ± 0.5**	0.03[b]

Note: Data are means ± SD. Fasting and 2-h insulin, pro-NT, triglycerides, AST, ALT, GGT, and hsCRP were log transformed for statistical analysis, but values in the table represent back transformation to the original scale. Categorical variables were compared by χ^2 test. Comparisons among the three groups were performed using a general linear model. p Values refer to results after analyses with adjustment for age, gender, and BMI.

[a] p Values refer to results after analyses with adjustment for gender and age.

[b] p Values refer to results after analyses with adjustment for gender and BMI.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FIB-4, Fibrosis 4; GGT, gamma-glutamyltransferase; HDL, high-density lipoprotein; hsCRP, high sensitivity C reactive protein; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NGT, normal glucose tolerance.

* $p < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus first tertile of pro-NT.

# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus second tertile of pro-NT.

p value less than 0.05 was considered statistically significant.

As no studies have investigated circulating pro-NT levels in relation to incident NAFLD, to assess the statistical power of this study, we performed a post hoc sample size calculation by using Gpower 3.1 program. Considering the means and SD of pro-NT concentrations that we detected in subjects with or without incident NAFLD, we calculated that 26 individuals for each group were enough to detect 70%–80% differences in pro-NT among study groups with a power of 90% and a level of significance of 5%.

Results

Cross-sectional association between pro-NT and NAFLD

The whole study population, comprising 303 subjects with a mean age of 44.6 ± 12.2 years, a mean BMI of 31.2 ± 6.7 kg/m^2, and mean pro-NT of 144.7 ± 130.7 pg/mL, was subdivided into tertiles according to serum levels of pro-NT. Clinical parameters of individuals in the lowest, intermediate, and highest pro-NT tertiles are shown in Table 1. No significant differences in term of age and gender were detected among the three study groups. Conversely, we found a progressive increase in BMI, waist circumference, and fat mass

in the intermediate and highest tertiles of pro-NT as compared to the lowest pro-NT group. After adjusting for age, gender, and BMI, we observed an association between pro-NT serum levels and a worse metabolic profile, with subjects in the intermediate and highest tertile of pro-NT showing progressively higher levels of triglycerides, 2-h post-load insulin levels, hsCRP, and decreased whole body insulin sensitivity estimated by Matsuda index, and HDL cholesterol concentrations as compared to the lowest tertile of pro-NT (Table 1). No significant difference in glucose tolerance was detected among the three study groups. Furthermore, after adjusting for age and gender, we found that higher levels of pro-NT were associated with augmented values of liver IR index, an index of hepatic insulin resistance. Accordingly, the proportion of subjects having ultrasound-defined NAFLD was progressively and significantly increased in the intermediate and highest pro-NT groups as compared to the lowest tertile of pro-NT (Table 1). Notably, subjects with higher serum concentrations of pro-NT showed significantly increased levels of the liver damage markers ALT and GGT, and FIB-4 index (Table 1).

In order to estimate the independent relationship between pro-NT serum levels and presence of NAFLD, we built a logistic regression model, including age, gender, BMI, total and HDL cholesterol, triglycerides, hsCRP, fasting, and 2-h post-load glucose and insulin levels. Individuals in the highest tertile of pro-NT displayed a significantly 3.43-fold higher risk of having NAFLD (OR: 3.43, 95%CI 1.48–7.97, $p = 0.004$) as compared to the lowest pro-NT group (Fig. 1). A no significant 1.67-fold increased risk was observed in subjects in the intermediate pro-NT tertile (OR: 1.67, 95%CI 0.73–3.84, $p = 0.06$) (Fig. 1). Similar results were found when BMI was replaced by waist circumference in the logistic regression analysis model, with subjects in the highest and intermediate tertile of pro-NT exhibiting, respectively, a 3.52-fold (OR: 3.52, 95%CI: 1.66–7.47, $p = 0.001$) and a 1.79-fold (OR: 1.79, 95%CI: 0.84–3.82, $p = 0.13$) increased risk of having NAFLD as compared to the lowest tertile of pro-NT.

Longitudinal association between pro-NT and incident NAFLD

Next, we tested the hypothesis that higher pro-NT levels may predict the development of NAFLD in study participants without ultrasound signs of NAFLD at baseline who were reexamined after a median follow-up of 5.1 ± 1.2 years ($n = 124$). This study cohort was subdivided according to incident NAFLD at follow-up into two groups: 58 subjects without incident NAFLD and 66 subjects with newly diagnosed NAFLD at follow-up. As compared to subjects without incident NAFLD, those developing NAFLD during follow-up period tended to have raised values of triglycerides, 2-h post-load glucose and insulin, hsCRP, ALT, and lower levels of HDL and Matsuda index at baseline, although no statistically significant difference in these metabolic parameters was detected between the two study groups (Table 2). Moreover, subjects developing NAFLD during the follow-up exhibited a significantly increased degree of hepatic insulin resistance assessed by liver IR index as compared to those without incident NAFLD. At follow-up visit, subjects with newly diagnosed NAFLD displayed higher levels of triglycerides, 2-h post-load glucose and insulin, liver IR index, and hepatic damage measures, such as AST, ALT, GGT, and FIB-4 index, and were more likely to have T2DM or IGT than those without NAFLD (Table 2). Notably, we found that subjects with incident NAFLD exhibited significantly higher levels of pro-NT at baseline as compared to those who were free from NAFLD (Fig. 2). Additionally, baseline pro-NT levels were positively correlated with hepatic fibrosis estimated by FIB-4 index at follow-up visit (Fig. 3).

To estimate the independent contribution of baseline serum pro-NT levels to the risk of developing

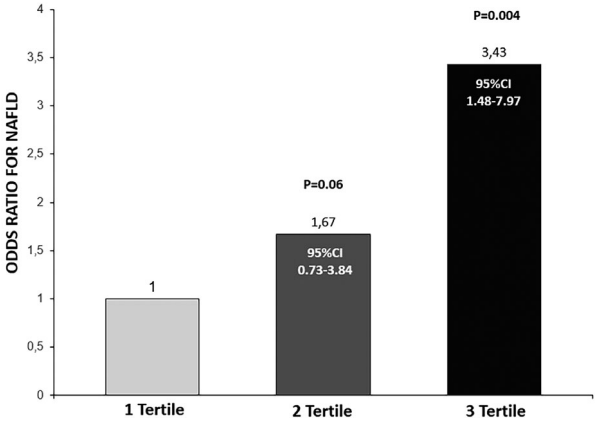

Fig. 1 *Risk of having nonalcoholic fatty liver disease (NAFLD) in subjects stratified into tertiles according to proneurotensin (pro-NT) levels in a logistic regression analysis model adjusted for age, gender, body mass index (BMI), total and HDL cholesterol, triglycerides, hsCRP, fasting, and 2-h post-load glucose and insulin levels.*

Table 2. *Clinical parameters of study subjects stratified according to incident ultrasonography-defined nonalcoholic fatty liver disease (NAFLD) at follow-up visit*

	Baseline			Follow-up		
	Without incident NAFLD	With incident NAFLD	*p*	Without incident NAFLD	With incident NAFLD	*p*
Gender (M/F)	25/33	32/34	0.54			
Age (years)	47.5 ± 12	48.4 ± 10	0.68	52.7 ± 12	53.1 ± 11	0.83
BMI (kg/m^2)	29.1 ± 5.0	28.9 ± 5.5	0.86	29.6 ± 4.4	30.2 ± 6.3	0.59
Waist circumference (cm)	98 ± 12	97 ± 11	0.59	100 ± 12	103 ± 13	0.22
Total cholesterol (mg/dL)	210 ± 38	209 ± 39	0.94	191 ± 34	201 ± 42	0.17
HDL cholesterol (mg/dL)	54 ± 15	49 ± 11	0.06	51 ± 11	48 ± 11	0.16
Triglycerides (mg/dL)	113 ± 49	124 ± 59	0.12	101 ± 43	122 ± 54	0.04
Fasting glucose (mg/dL)	92 ± 9	92 ± 11	0.90	92 ± 9	95 ± 15	0.18
2-h post-load glucose (mg/dL)	111 ± 30	118 ± 26	0.23	114 ± 26	137 ± 38	<0.0001
Fasting insulin (mU/mL)	12 ± 7	13 ± 8	0.35	15 ± 19	16 ± 13	0.63
2-h post-load insulin (mU/mL)	69 ± 56	85 ± 63	0.17	66 ± 41	126 ± 115	0.01
NGT/IFG/IGT/IFG + IGT/T2DM	45/8/1/4/0	44/12/7/3/0	0.17	40/8/3/4/3	34/7/11/5/9	0.03
AST (Ul/L)	20 ± 7	21 ± 7	0.38	20 ± 8	25 ± 17	0.03
ALT (Ul/L)	22 ± 10	26 ± 13	0.09	20 ± 6	27 ± 20	0.01
GGT (Ul/L)	20 ± 11	28 ± 23	0.21	19 ± 9	32 ± 29	<0.0001
hsCRP (mg/L)	2.5 ± 3.0	3.1 ± 4.4	0.21	2.6 ± 2.5	3.0 ± 2.8	0.76
Pro-NT (pg/mL)	60 ± 50	115 ± 80	0.01			
Matsuda index	4.1 ± 3.1	3.3 ± 2.5	0.23	2.3 ± 2.1	1.7 ± 1.1	0.20
Liver IR index	2.3 ± 0.3	2.6 ± 0.4	0.04	2.8 ± 0.3	3.1 ± 0.4	0.01
FIB-4 index	0.82 ± 0.3	0.84 ± 0.3	0.80	1.01 ± 0.4	1.21 ± 0.5	0.03

Note: Data are presented as means ± SD. Fasting, 2-h post-load insulin, pro-NT, triglycerides, AST, ALT, GGT, and hsCRP levels were log transformed for statistical analysis, but values in the table represent back transformation to the original scale. Categorical variables were compared by χ^2 test. Continuous variables were compared using a Student *t* test.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FIB-4, Fibrosis 4; GGT, gamma-glutamyltransferase; HDL-C, high-density lipoprotein-cholesterol; hsCRP, high-sensitivity C-reactive protein; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; pro-NT, proneurotensin; T2DM, type 2 diabetes.

NAFLD at follow-up, we built five models of Cox hazard regression analysis of growing complexity (Table 3). In model 1, including age, gender, BMI, total cholesterol, triglycerides, HDL cholesterol, fasting, and 2-h post-load glucose and insulin, and hsCRP as covariates, we found that each SD increase of pro-NT was associated with a 1.69-fold increased HR (95%CI 1.20–2.38) of future NAFLD. Baseline pro-NT levels were a predictor of incident NAFLD even when BMI was replaced by waist circumference, and Matsuda index was included in the Cox regression model 2. When BMI, total cholesterol, triglycerides, and HDL cholesterol gathered at follow-up visit were included in the Cox regression model 3, higher pro-NT levels at baseline remained an independent predictor of incident NAFLD (Table 3). Similar results were found after further adjustment for newly diagnosed type 2 diabetes at follow-up (model 4). Alternatively, when fasting and 2-h post-load glucose levels at follow-up visit were included in the Cox regression model (model 5), pro-NT levels at baseline were independently associated with an increased risk of incident NAFLD, along with 2-h post-load glucose and triglycerides at follow-up (Table 3).

Conclusions

It is broadly recognized that the pathogenesis of NAFLD is complex and multifactorial, encompassing several alterations, most of them also implicated in the pathogenesis of obesity and T2DM, such as unhealthy lifestyle, adipose tissue dysfunction, inflammation, insulin resistance, altered gut microbiota, and permeability, others not directly linked to a worse cardiovascular risk

Table 3. *Cox regression analysis of incident nonalcoholic fatty liver disease (NAFLD)*

	Independent predictors	HR	95%CI	*p*
Model 1: Gender, age, BMI, total cholesterol, triglycerides, HDL cholesterol, hsCRP, fasting, and 2-h post-load glucose and insulin	pro-NT	1.69	1.203–2.383	0.01
Model 2: Gender, age, waist circumference, total cholesterol, triglycerides, HDL cholesterol, hsCRP, Matsuda index	pro-NT	1.75	1.225–2.513	0.01
Model 3: Model 1 + BMI, total cholesterol, triglycerides, HDL cholesterol at follow-up visit	pro-NT	1.54	1.065–2.224	0.02
	Triglycerides at follow-up visit	1.018	1.003–1.033	0.02
Model 4: Model 3 + newly diagnosed type 2 diabetes at follow-up visit	pro-NT	1.54	1.065–2.224	0.02
	Triglycerides at follow-up visit	1.018	1.003–1.033	0.02
Model 5: Model 3 + fasting and 2-h post-load glucose at follow-up visit	pro-NT	1.52	1.017–2.282	0.04
	2-h post-load glucose at follow-up	1.025	1.004–1.047	0.02
	Triglycerides at follow-up visit	1.017	1.001–1.034	0.04

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; pro-NT, proneurotensin.

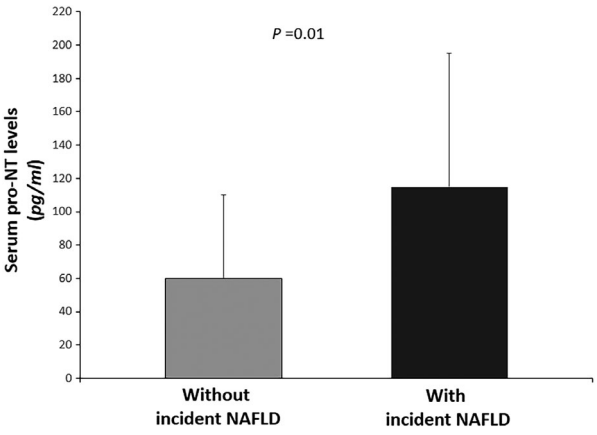

Fig. 2 *Fasting serum levels of proneurotensin (pro-NT) at baseline in subjects with or without newly diagnosed nonalcoholic fatty liver disease (NAFLD) at follow-up visit.*

profile, including altered lipoprotein biosynthesis and mobilization [34, 41, 42]. Despite the intense research efforts, pathophysiologic players involved in the development and progression of NAFLD have not been completely identified yet. Given the growing diffusion of NAFLD worldwide and its hepatic and extrahepatic complications, understanding the pathogenesis of NAFLD, in order to identify potential biomarkers recognizing high-risk subjects and novel therapeutic target, represents a major goal.

Accumulating evidence has indicated the role of the gut hormone NT in the pathogenesis of obesity and its related comorbidities, including T2DM and NAFLD [8, 10–15, 17–20, 31–33]. Indeed, previous experimental studies have demonstrated that NT is involved in body weight control both by modulating brain circuits implicated in the regulation of food intake and energy expenditure and by promoting gut lipid absorption [11–13]. Moreover, several preclinical evidence indicates that, in addition to control energy homeostasis, NT may exert pro-inflammatory activities, promote ectopic fat deposition, and alter gut microbiota and intestinal barrier integrity [15, 18, 22–24, 43]. Interestingly, genetic or pharmacological inhibition of NT/NTRs axis has been found to counteract high-fat diet-induced hepatic steatosis in mice [12–13, 15, 44]. However, although several preclinical evidence has demonstrated a pathogenic role of NT in development and progression of NAFLD, a few human

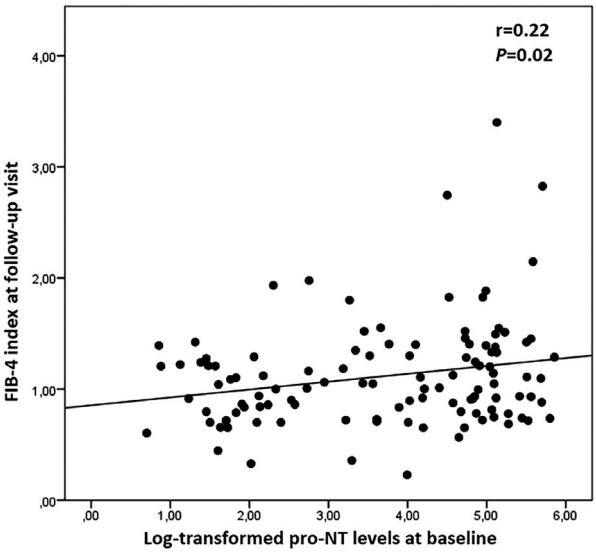

Fig. 3 *Univariate analysis between fasting serum levels of proneurotensin (pro-NT) at baseline (naturally log transformed) and Fibrosis-4 (FIB-4) index at follow-up visit.*

studies, mainly carried out on a small number of morbidly obese subjects, have investigated the association between increased levels of NT and NAFLD [31–33]. Additionally, even though higher circulating pro-NT levels have been reported to predict obesity, T2DM, breast cancer, total and cardiovascular mortality [12, 17, 19–21], no longitudinal studies have been carried out to explore whether increased NT levels may independently predict NAFLD development. In the present study, we provide evidence that higher levels of pro-NT, which is an indicator of NT secretion, are associated with and predict the development of NAFLD independently of other metabolic risk factors using two distinct approaches. First, we carried out a cross-sectional analysis in order to evaluate the association between circulating pro-NT levels and prevalent ultrasound-defined NAFLD in nondiabetic subjects. Stratifying our study population into tertile of pro-NT levels, we found that higher pro-NT levels were associated with NAFLD and raised levels of FIB-4 index, a validated estimate of an increased risk of hepatic fibrosis. By performing a logistic regression analysis, we found that individuals with higher pro-NT levels exhibit an increased risk of having NAFLD as compared to individuals in the lowest pro-NT tertile independently of several potential confounders such as age, gender, BMI, total and HDL cholesterol, triglycerides, hsCRP, fasting, and 2-h post-load glucose and insulin levels. These results, confirming and extending those reported by other authors [31, 32], indicate that raised levels of circulating pro-NT confer an augmented risk of NAFLD regardless demographic and anthropometric measures, pro-inflammatory and lipid profile, and glucose homeostasis parameters. Moreover, individuals with higher pro-NT levels exhibit increased BMI, waist circumference, and fat mass than those with lower pro-NT levels, confirming the relationship between augmented pro-NT/NT concentrations and increased adiposity as previously described [11, 12, 18, 19]. Remarkably, even after adjusting for BMI, in addition to age and gender, we found that increased levels of pro-NT are associated with an unfavorable metabolic profile, including higher levels triglycerides, hsCRP, 2-h post-load insulin, hepatic insulin resistance, and lower values of HDL and peripheral insulin sensitivity estimated by the Matsuda index. These findings, coupled with prior preclinical data demonstrating the capability of NT to affect lipid metabolism, insulin responsiveness, and stimulate pro-inflammatory pathways [11, 12, 18, 22–24], support the idea that NT may directly induce a metabolic impairment independently of adiposity measures.

Second, in an attempt to investigate the causal relationship between higher pro-NT levels and NAFLD development, we assessed the association between pro-NT levels and incident NAFLD in a subgroup of subjects without NAFLD at baseline who underwent to a careful characterization after 5 years follow-up. We found, for the first time, that subjects with newly diagnosed NAFLD at follow-up visit have significantly higher levels of pro-NT at baseline. Interestingly, by performing a multivariate Cox regression analysis, we observed that baseline pro-NT levels were a predictor of future development of NAFLD independently of other baseline metabolic parameters. Because prior studies have provided evidence that raised pro-NT levels predict the development of obesity, T2DM, and metabolic syndrome [12, 17–21], all conditions widely known to be related to NAFLD, it is plausible to hypothesize an indirect effect of augmented pro-NT levels on NAFLD development mediated by the worsening of glucose tolerance, lipid metabolism, and adiposity during the follow-up. However, our results argue against this possibility. Indeed, when BMI, lipid profile, and glucose homeostasis parameters collected at follow-up visit were included in the Cox regression model, the association between baseline pro-NT levels and incident NAFLD was retained,

suggesting that raised levels of pro-NT are an independent risk factor for the development of NAFLD. Additionally we observed a positive association between baseline pro-NT and hepatic fibrosis risk at follow-up visit, estimated by FIB-4 index.

Altogether the present findings, demonstrating the independent association of higher pro-NT levels with both prevalent and incident NAFLD, support the notion that the determination of pro-NT levels may be a valuable tool to identify subjects at increased risk of having or developing NAFLD. Additionally, our results coupled with prior evidence of beneficial effects of strategies blocking pro-NT/NTRs on liver histology in animal models of obesity [12, 13, 15, 44] lay the basis for future studies testing the intriguing possibility that NT may be a pharmacological target for prevention and treatment of NAFLD in humans.

Our study has several straights including the longitudinal design with the accurate metabolic characterization of the study population, the inclusion of both men and women, the centralization of biochemical analyses, the ultrasound diagnosis of NAFLD performed by an experienced examiner who was blinded to the clinical data of study subjects, the exclusion of confounding conditions potentially, inducing liver damage, such as drug abuse, positivity for antibodies to HCV or HBsAg, and T2DM. Nevertheless, a number of weaknesses need to be considered. First, the relatively limited sample size may represent a limitation of our study, although power calculation allows us to exclude type I error. Notably, it should be considered that all study participants underwent to a detailed physical and biochemical evaluation both at baseline and follow-up visit, which is not easily feasible in large observational studies. Second, diagnosis of NAFLD was established by hepatic ultrasound-based criteria rather than more sensitive but also invasive and expensive approaches, such as liver biopsy, proton magnetic resonance spectroscopy, or computed tomographic scanning. Moreover, our study population encompasses adult White subjects, and whether the present findings may be extended to other ethnic groups should be verified. Furthermore, the evidence of a longitudinal association between raised pro-NT levels and NAFLD suggest but not firmly prove the causal relationship between an upregulated NT axis and NAFLD development. We cannot exclude the effect of other potential confounders, including dietary intake of lipid or sugars, which stimulate NT secretion and may directly contribute to liver damage [45, 46], or genetic/epigenetic factors [42] not explored in this study.

In conclusion, our data demonstrate that increased pro-NT levels are an independent predictor of NAFLD, suggesting that the determination of circulating pro-NT levels may be a potential screening tool to identify subjects at increased risk of having or developing NAFLD and support the view that the upregulated NT/NTR axis may promote liver damage and represent a therapeutic target for NAFLD development and progression.

Author contributions

Francesca De Vito performed ELISA assay, researched and analyzed data, wrote and edited the manuscript; Velia Cassano and Elettra Mancuso researched data and edited the manuscript; Elena Succurro, Angela Sciacqua, Marta Letizia Hribal, and Francesco Andreozzi researched data and reviewed the manuscript; Giorgio Sesti conceived the study, contributed to analyze data, and reviewed the manuscript; Teresa Vanessa Fiorentino researched, analyzed and interpreted data, contribute to wrote and edited the manuscript. All authors have read and approved the final manuscript.

Conflict of interest statement

The authors declare that they have no financial and nonfinancial conflict of interests.

Data availability statement

Data of study participants are available upon request.

References

1 Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*. 2016;**64**:73–84.
2 Younossi Z, Tacke F, Arrese M, Chander Sharma B, Mostafa I, Bugianesi E, et al. Global perspectives on nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Hepatology*. 2019;**69**(6):2672–82.
3 Perumpail BJ, Khan MA, Yoo ER, Cholankeril G, Kim D, Ahmed A. Clinical epidemiology and disease burden of nonalcoholic fatty liver disease. *World J Gastroenterol*. 2017;**23**:8263–76.
4 Fiorentino TV, Succurro E, Sciacqua A, Andreozzi F, Perticone F, Sesti G. Non-alcoholic fatty liver disease is associated with cardiovascular disease in subjects with different glucose tolerance. *Diabetes Metab Res Rev*. 2020;**36**(8):e3333.

5 Duell PB, Welty FK, Miller M, Chait A, Hammond G, Ahmad Z, et al. Nonalcoholic fatty liver disease and cardiovascular risk: a scientific statement from the American Heart Association. *Arterioscler Thromb Vasc Biol.* 2022;**42**(6):e168–85.
6 Mantovani A, Scorletti E, Mosca A, Alisi A, Byrne CD, Targher G. Complications, morbidity and mortality of nonalcoholic fatty liver disease. *Metabolism* 2020;**111S**:154170.
7 Polak JM, Sullivan SN, Bloom SR, Buchan AMJ, Facer P, Brown MR, et al. Specific localisation of neurotensin to the N cell in human intestine by radioimmunoassay and immunocytochemistry. *Nature.* 1977;**270**(5633):183–4.
8 Grunddal KV, Ratner CF, Svendsen B, Sommer F, Engelstoft MS, Madsen AN, et al. Neurotensin is coexpressed, coreleased, and acts together with GLP-1 and PYY in enteroendocrine control of metabolism. *Endocrinology* 2016;**157**:176–94
9 Kalafatakis K, Triantafyllou K. Contribution of neurotensin in the immune and neuroendocrine modulation of normal and abnormal enteric function. *Regul Pept.* 2011;**170**:7–17
10 Mazella J, Béraud-Dufour S, Devader C, Massa F, Coppola T. Neurotensin and its receptors in the control of glucose homeostasis. *Front Endocrinol.* 2012;**3**:143
11 Barchetta I, Baroni MG, Melander O, Cavallo MG. New insights in the control of fat homeostasis: the role of neurotensin. *Int J Mol Sci.* 2022;**23**(4):2209
12 Li J, Song J, Zaytseva YY, Liu Y, Rychahou P, Jiang K, et al. An obligatory role for neurotensin in high-fat-diet-induced obesity. *Nature.* 2016;**533**:411–5.
13 Rabinowich L, Fishman S, Hubel E, Thurm T, Park W-J, Pewzner-Jung Y, et al. Sortilin deficiency improves the metabolic phenotype and reduces hepatic steatosis of mice subjected to diet-induced obesity. *J Hepatol.* 2015;**62**(1):175–81.
14 Vincent J-P, Mazella J, Kitabgi P. Neurotensin and neurotensin receptors. *Trends Pharmacol Sci.* 1999;**20**:302–9.
15 Chen C, Li J, Matye DJ, Wang Y, Li T. Hepatocyte sortilin 1 knockout and treatment with a sortilin 1 inhibitor reduced plasma cholesterol in Western diet-fed mice. *J Lipid Res.* 2019;**60**(3):539–49.
16 Ernst A, Hellmich S, Bergmann A. Proneurotensin 1–117, a stable neurotensin precursor fragment identified in human circulation. *Peptides.* 2006;**27**:1787–93.
17 Barchetta I, Bertoccini L, Sentinelli F, Bailetti D, Marini G, Cimini FA, et al. Circulating pro-neurotensin levels predict bodyweight gain and metabolic alterations in children. *Nutr Metab Cardiovasc Dis.* 2021;**31**:902–10.
18 Barchetta I, Cimini F, Capoccia D, Bertoccini L, Ceccarelli V, Chiappetta C, et al. Neurotensin is a lipid-induced gastrointestinal peptide associated with visceral adipose tissue inflammation in obesity. *Nutrients.* 2018;**10**(4):526.
19 Nicoli CD, Carson AP, Plante TB, Leann Long D, Mcclure LA, Schulte J, et al. Pro-neurotensin/neuromedin N and risk of incident metabolic syndrome and diabetes mellitus in the REGARDS Cohort. *J Clin Endocrinol Metab.* 2021;**106**(9):e3483–94.
20 Melander O, Maisel AS, Almgren P, Manjer J, Belting M, Hedblad Bo, et al. Plasma proneurotensin and incidence of diabetes, cardiovascular disease, breast cancer, and mortality. *JAMA.* 2012;**308**:1469–75.
21 Januzzi JL, Lyass A, Liu Y, Gaggin H, Trebnick A, Maisel AS, et al. Circulating proneurotensin concentrations and cardiovascular disease events in the community: the Framingham Heart Study. *Arterioscler Thromb Vasc Biol.* 2016;**36**:1692–7.
22 Castagliuolo I, Wang C-C, Valenick L, Pasha A, Nikulasson S, Carraway RE, et al. Neurotensin is a proinflammatory neuropeptide in colonic inflammation. *J Clin Invest.* 1999;**103**:843–9.
23 Zhao D, Keates AC, Kuhnt-Moore S, Moyer MP, Kelly CP, Pothoulakis C. Signal transduction pathways mediating neurotensin-stimulated interleukin-8 expression in human colonocytes. *J Biol Chem.* 2001;**276**:44464–71.
24 Koon H-W, Kim YS, Xu H, Kumar A, Zhao D, Karagiannides I, et al. Neurotensin induces IL-6 secretion in mouse preadipocytes and adipose tissues during 2,4,6,-trinitrobenzensulphonic acid-induced colitis. *Proc Natl Acad Sci USA.* 2009;**106**:8766–71.
25 Patel AB, Tsilioni I, Leeman SE, Theoharides TC. Neurotensin stimulates sortilin and mTOR in human microglia inhibitable by methoxyluteolin, a potential therapeutic target for autism. *Proc Natl Acad Sci USA.* 2016;**113**:E7049–58.
26 Riehle KJ, Kenerson HL, Riggle KM, Turnham R, Sullivan K, Bauer R, et al. Neurotensin as a source of cyclic AMP and co-mitogen in fibrolamellar hepatocellular carcinoma. *Oncotarget.* 2019;**10**:5092–102.
27 Xiao P, Long X, Zhang L, Ye Y, Guo J, Liu P, et al. Neurotensin/IL-8 pathway orchestrates local inflammatory response and tumor invasion by inducing M2 polarization of Tumor-Associated macrophages and epithelial-mesenchymal transition of hepatocellular carcinoma cells. *Oncoimmunology.* 2018;**7**:e1440166.
28 Browning JD, Horton JD. Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest.* 2004;**114**:147–52.
29 Lv Q, Zhen Q, Liu L, Gao R, Yang S, Zhou H, et al. AMP-kinase pathway is involved in tumor necrosis factor alpha-induced lipid accumulation in human hepatoma cells. *Life Sci.* 2015;**131**:23–9.
30 Han J, Wang Y. mTORC1 signaling in hepatic lipid metabolism. *Protein Cell.* 2018;**9**:145–51.
31 Barchetta I, Cimini FA, Leonetti F, Capoccia D, Di Cristofano C, Silecchia G, et al. Increased plasma proneurotensin levels identify NAFLD in adults with and without type 2 diabetes. *J Clin Endocrinol Metab.* 2018;**103**:2253–60.
32 Villar B, Bertran L, Aguilar C, Binetti J, Martínez S, Sabench F, et al. Circulating levels of pro-neurotensin and its relationship with nonalcoholic steatohepatitis and hepatic lipid metabolism. *Metabolites.* 2021;**11**:373.
33 Dongiovanni P, Meroni M, Petta S, Longo M, Alisi A, Soardo G, et al. Neurotensin up-regulation is associated with advanced fibrosis and hepatocellular carcinoma in patients with MAFLD. *Biochim Biophys Acta Mol Cell Biol Lipids.* 2020;**1865**:158765.
34 Hazlehurst JM, Woods C, Marjot T, Cobbold JF, Tomlinson JW. Non-alcoholic fatty liver disease and diabetes. *Metabolism.* 2016;**65**(8):1096–108.
35 Fiorentino TV, Succurro E, Marini MA, Pedace E, Andreozzi F, Perticone M, et al. HDL cholesterol is an independent predictor of β-cell function decline and incident type 2 diabetes: a longitudinal study. *Diabetes Metab Res Rev.* 2020;**36**(4):e3289.
36 Fiorentino TV, Miceli S, Succurro E, Sciacqua A, Andreozzi F, Sesti G. Nonalcoholic fatty liver disease is associated with a decreased myocardial mechano-energetic efficiency. *J Intern Med.* 2021;**289**(2):221–31.

37 American Diabetes Association. 2. Classification and diagnosis of diabetes: standards of medical care in diabetes-2020. *Diabetes Care.* 2020;**43**:S14–31.
38 Matsuda M, Defronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care.* 1999;**22**:1462–70.
39 Vangipurapu J, Stančáková A, Kuulasmaa T, Paananen J, Kuusisto J, Ferrannini E, et al. A novel surrogate index for hepatic insulin resistance. *Diabetologia.* 2011;**54**:540–3.
40 Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, et al. The diagnosis and management of nonalcoholic fatty liver disease: practice guidance from the American Association for the Study of Liver Diseases. *Hepatology.* 2018;**67**:328–57.
41 Buzzetti E, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metabolism.* 2016;**65**(8):1038–48.
42 Stefan N, Cusi K. A global view of the interplay between non-alcoholic fatty liver disease and diabetes. *Lancet Diabetes Endocrinol.* 2022;**10**(4):284–96.
43 Li J, Li X, Song J, Yan B, Rock SA, Jia J, Liu J, et al. Absence of neurotensin attenuates intestinal dysbiosis and inflammation by maintaining Mmp7/α-defensin axis in diet-induced obese mice. *FASEB J.* 2020;**34**:8596–610.
44 Wu Z, Stadler N, Abbaci A, Liu J, Boullier A, Marie N, et al. Effect of monoclonal antibody blockade of long fragment neurotensin on weight loss, behavior, and metabolic traits after high-fat diet induced obesity. *Front Endocrinol (Lausanne).* 2021;**12**:739287.
45 Drewe J, Mihailovic S, D'amato M, Beglinger C. Regulation of fat-stimulated neurotensin secretion in healthy subjects. *J Clin Endocrinol Metab.* 2008;**93**:1964–70.
46 Kuhre RE, Bechmann LE, Wewer Albrechtsen NJ, Hartmann B, Holst JJ. Glucose stimulates neurotensin secretion from the rat small intestine by mechanisms involving SGLT1 and GLUT2, leading to cell depolarization and calcium influx. *Am J Physiol Endocrinol Metab.* 2015;**308**(12):E1123–30.

Correspondence:
Giorgio Sesti, Department of Clinical and Molecular Medicine, University of Rome-Sapienza, Rome 00189, Italy.
Email: giorgio.sesti@uniroma1.it