Increased plasma proneurotensin levels identify NAFLD in adults with and without type 2 diabetes

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Proneurotensin and NAFLD in diabetes

Increased plasma proneurotensin levels identify NAFLD in adults with and without type 2 diabetes

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List of Abbreviations

NT: Neurotensin

NAFLD: non-alcoholic fatty liver disease

NASH: steatohepatitis

Pro-NT: proneurotensin 1-117

T2D: type 2 diabetes

BMI: body mass index

SBP systolic blood pressure

DBP: diastolic blood pressure

OGTT: oral glucose tolerance test

FBG: Fasting blood glucose

HbA1c: glycosylated hemoglobin

HDL: high-density lipoprotein cholesterol

AST: aspartate aminotransferase

ALT: alanine aminotransferase

FBI: fasting blood insulin

LDL: low-density lipoprotein cholesterol

HOMA-IR: insulin resistance

HOMA-β: homeostasis model assessment of insulin secretion

NAS: NAFLD activity score

SAF: Steatosis Activity Fibrosis score

AUROC: area under receiver-operating characteristic curve

Context Neurotensin (NT) is an intestinal peptide released by fat ingestion and promoting lipids absorption; higher circulating NT levels associate with type 2 diabetes (T2D), obesity and cardiovascular disease. Whether NT is related to non-alcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH) has not been fully investigated.

Objective To study the relationship between plasma proneurotensin1-117 (pro-NT), a stable fragment of the NT precursor hormone, and the presence/severity of NAFLD/NASH and to unravel correlates of increased pro-NT levels.

Design/Setting/Participants For this cross-sectional study, sixty obese individuals undergoing bariatric surgery for clinical purposes were recruited. The association between pro-NT and NAFLD was further investigated in 260 consecutive subjects referring to our outpatient clinics for metabolic evaluations including liver ultrasonography. Study population underwent complete metabolic characterization; in the obese cohort, liver biopsies were performed during surgery.
Main outcome measures Plasma pro-NT levels in relation to NAFLD/NASH.
Results Obese subjects with biopsy-proven NAFLD (53%) had significantly higher plasma pro-NT than those without NAFLD (183.6±81.4 vs 86.7±56.8 pmol/L, p<0.001). Greater pro-NT correlated with NAFLD presence (p<0.001) and severity (p<0.001), age, female gender, insulin-resistance and T2D. Higher pro-NT predicted NAFLD with AUROC=0.836 (C.I.95%:0.73-0.94, p<0.001). Belonging to the highest pro-NT quartile correlated with increased NAFLD risk (OR:2.62; 95%CI:1.08-6.40), after adjustment for confounders. The association between higher pro-NT and NAFLD was confirmed in the second cohort, independently from confounders.
Conclusions Increased plasma pro-NT levels identify the presence/severity of NAFLD; in dysmetabolic individuals, NT may specifically promote hepatic fat accumulation though mechanisms likely related to increased insulin-resistance.

This study shows for the first time in humans the existence of a correlation between circulating neurotensin and biopsy-proven NAFLD/NASH.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a pathological condition characterized by the macrovesicular accumulation of triglycerides within hepatocytes (hepatic steatosis); in a number of cases, necro-inflammatory activity and fibrosis coexist (non-alcoholic steatohepatitis, NASH); furthermore, cirrhosis and liver failure may occur in 20-25% of affected individuals (1, 2). Nowadays, NAFLD represents the most common cause of chronic liver disease in developed countries (3), being detectable in 20-30% of general population (4, 5) in almost 75% of type 2 diabetic (T2D) patients (6, 7) and in up to 90% obese T2D individuals (8, 9). In dysmetabolic conditions, NAFLD worsens inflammatory and metabolic outcomes (10-12) and is associated with greater prevalence and severity of micro and macro-vascular complications in patients with T2D (13-15). Indeed, NAFLD is universally recognized as an independent risk factor for cardiovascular mortality (16). Nowadays, despite the impressively high number of pharmacological interventions proposed, the identification of an effective therapy of NAFLD beyond standard lifestyle measures, is still an open issue and represents a major challenge (17).

Neurotensin (NT) is a 13 amino-acid peptide mainly secreted by neuroendocrine cells in the small intestine tract (18) and displays an important role in regulating food ingestion and fat absorption (19). By doing so, NT influences energy balance and body weight (20). 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, NTSR1, 2 and 3 (21, 22). Experimental evidence has shown that NT/NTSR1 system is involved in the adaptive energy balance (23-25); NTSR1 loss of function determined overweight and impaired ability to appropriately respond to energy deprivation signals (24), pointing out to a crucial role of NT in mediating, among others, leptin (23-25) and ghrelin (25) pathways. Indeed, the leptin-mediated systems regulating appetite are controlled by NT expressing neurons (23). In the periphery, NT influences body weight by controlling macronutrients absorption. Physiology studies described acute increase of intestine NT release immediately after food ingestion, directly associated with meal fat content (26). Later on, several data have been produced on the role of NT in facilitating lipid digestion and fat absorption in the

small intestine (27-29). The refined, complex control of energy balance exerted by NT at different levels, provides a possible pathophysiological explanation to the evidence of a correlation between its circulating levels and increased prevalence and incidence of obesity-related diseases (30, 31). In particular, within the large cohort of the Malmö Diet and Cancer Study (30), fasting concentration of pro-NT, the circulating peptide secreted at equimolar levels to NT, was associated with the incidence of T2D, cardiovascular disease, breast cancer, and with total and cardiovascular mortality (30). The association between pro-NT and incident major cardiovascular events was, lately, confirmed in the Framingham Heart Study Offspring cohort, independently of the presence of traditional cardiovascular risk factors (31). Very recently, an extensive investigation on a putative causal role of NT in determining aberrant fat accumulation and metabolic diseases has been carried out (29), showing reduced intestinal fat absorption, along with protection from obesity and NAFLD, in NT-deficient mice fed with high fat diet. Furthermore, the same study demonstrated that, in humans, higher plasma pro-NT levels were associated with features of insulin resistance and doubled the risk of developing obesity later in life non-obese subjects.

Despite the strong rationale behind and encouraging evidence from animal models, little is known on circulating pro-NT levels and NAFLD/NASH in humans. Therefore, aims of this study were to investigate the relationship between plasma NT concentration and the presence and severity of NAFLD/NASH in adult obese individuals with or without T2D and to determine clinical correlates of impaired NT levels in this population.

Materials and Methods

Population

For these purposes, we recruited sixty consecutive obese candidates to bariatric surgery referring to the Endocrinology and Diabetes outpatient clinics of Sapienza University of Rome for pre-operative evaluations. The presence of an association between circulating pro-NT levels and NAFLD was further explored in a cohort of individuals (n= 260) referring to the same outpatient clinics for metabolic evaluations, including upper abdomen US for assessing the presence of fatty liver. To be eligible for the study, all the study participants had to fulfil the following criteria: male and female aged between 20 and 65 years, no history of current or past excessive alcohol drinking, as defined by an average daily consumption of alcohol > 30 gr/day in men and > 20 gr/day in women; negative tests for the presence of hepatitis B surface antigen and antibody to hepatitis C virus; absence of history and findings consistent with cirrhosis and other causes of liver diseases (autoimmune hepatitis, hemochromatosis, Wilson's disease), no treatment with drugs known to cause liver steatosis (e.g., corticosteroids, estrogens, methotrexate, tetracycline, calcium channel blockers, or amiodarone). Furthermore, patients belonging to the morbidly obese cohort, had clinical indication to bariatric surgery. All the participants underwent a complete work-up including medical history collection, clinical examination, anthropometric measurements and laboratory tests.

Clinical and laboratory assessment

Weight and height were measured with patients wearing light clothing and no shoes. The body mass index (BMI, kg/m^2) was calculated as weight in kilograms divided by the square of the height in meters. Waist circumference was measured midway between the 12th rib and the iliac crest. Systemic systolic (SBP) and diastolic (DBP) blood pressure were measured after 5 minutes resting; three measurements were taken and the average of the second and third measurements was recorded and used in the analyses. Individuals without a previously formulated diagnosis of diabetes mellitus underwent standard oral glucose tolerance test (OGTT) measuring blood glucose and insulin at baseline and 30, 60, 90 and 120 minutes after glucose ingestion. A 12-hour overnight fasting blood sample was obtained before

surgery for metabolic profiling. Fasting blood glucose (FBG, mg/dL), glycosylated hemoglobin (HbA1c, % - mmol/mol), total cholesterol (mg/dL), high-density lipoprotein cholesterol (HDL, mg/dL), triglycerides (mg/dL), aspartate aminotransferase (AST, IU/L), alanine aminotransferase (ALT, IU/L) and creatinine (mg/dL) were measured by centralized standard methods. Insulin (FBI, lU/mL) was measured by radioimmunoassay (ADVIA Insulin Ready Pack 100; Bayer Diagnostics, Milan, Italy), with intra- and inter-assay coefficients of variation <5%. Low-density lipoprotein (LDL) cholesterol value was obtained using Friedwald formula.

For our purposes, we measured circulating concentration of pro-NT on plasma frozen immediately after separation and stored at -80°C. Pro-NT was measured using a chemiluminometric sandwich immunoassay to detect pro-NT amino acids 1–117 as described previously (32). The analytical assay sensitivity (mean relative light units of 10 determinations sheep serum plus 2 standard deviations) was 4.8 pmol proNT/l. The inter assay (10 assay runs) coefficient of variability was 6.2% at 48 pmol proNT/l and 4,1% at 191 pmol/L. Recovery and dilution was > 85% in a measurement range of 25-850 pmol/l.

The homeostasis model assessment of insulin resistance (HOMA-IR) and insulin secretion (HOMA-β %) were calculated as previously described (33). Diabetes mellitus was defined according to the American Diabetes Association 2009 criteria (34) and metabolic syndrome (MS) by the modified NCEP ATP-III criteria (35).

Liver biopsy and histology

Study patients underwent intraoperative liver biopsy during surgery for sleeve gastrectomy. All the procedures were conducted in accordance with recommendations set by the American Association for the Study of Liver Diseases (36). Liver fragments were fixed in buffered formalin for 2-4 hours and embedded in paraffin, sections were cut and stained with hematoxylin and eosin and Masson's trichrome stains. A single pathologist blinded to patients' medical history and biochemistry performed the overall histological evaluations. A minimum biopsy length of 15 mm or the presence of 10 complete portal tracts was required (37). Liver biopsy samples were classified based on the presence of NASH by Brunt definition (38) and graded according to the NAFLD activity score (NAS) (39); fibrosis was quantified on the basis of the NASH Clinical Research Network Scoring System Definition (39).

NAFLD assessment in individuals not candidate to surgery

In individuals not candidate to surgery, NAFLD was evaluated through liver ultrasonography (US). This was performed using an Esaote Medica instrument with a convex 3,5 MHz probe by the same operator blinded to laboratory values. Liver steatosis was defined according to Saverymuttu et al. (40) on the basis of abnormally intense, high level echoes arising from the hepatic parenchyma, liver-kidney difference in echo amplitude, echo penetration into deep portion of the liver and clarity of liver blood vessel structure.

Statistics

SPSS version 23 was used to perform statistical analyses. Continuous variables are reported as the mean ± standard deviation (SD), and categorical variables are reported as percentages. Student's T-test for continuous variables, χ^2 test for categorical variables were used to compare mean values between two independent groups; skewed variables underwent natural logarithmic transformations before performing the analyses. Correlations between parameters were explored by Pearson's (continuous variables) or Spearman's (categorical variables) coefficients or by age-, gender-and BMI adjusted partial correlations. Histological parameters are expressed by ordinal scales for NAFLD Activity Score (NAS) and Steatosis Activity Fibrosis score (SAF) (38). NAS score was use as continuous scale for activity assessment, comparisons between >2 were obtained by Bonferroni-adjusted ANOVA test. The predictive

value of plasma pro-NT for NAFLD identification was estimated by the area under receiver-operating characteristic curve (AUROC), with a 95% confidence interval (C.I.). Multivariate logistic regression models were built to identify determinants of NAFLD (yes/no, dependent variable) in our study population, entering all the variables significantly associated at the bivariate analyses as covariates. Data are shown as mean ± standard deviation. For all the above, A two-tailed P value < 0.05 was considered statistically significant.

At the best of our knowledge, no study has investigated so far circulating pro-NT levels in relation to NAFLD. Therefore, in order to confirm the statistical power of this study, we performed a *post-hoc* sample size calculation considering mean pro-NT concentration in subjects with and without NAFLD and we obtained that fifteen patients per subgroup would have been enough to reach the statistical significance with power= 90% and α error= 0.05. For all the above, p value < 0.05 was considered statistically significant.

The study protocol was reviewed and approved by the Ethics Committee of Policlinico Umberto I, Sapienza University of Rome and conducted in conformance with the Helsinki Declaration. Written consent was obtained from all patients before the study.

Results

Pro-NT and biopsy-proven NAFLD/NASH

Within our study population, thirty-two subjects out of sixty subjects (53%) had histological features of NAFLD; clinical and biochemical characteristics of the study population in relation to the presence of NAFLD are shown in Table 1, along with results from age-, gender- and BMI-adjusted partial correlation analyses.

Plasma pro-NT levels were significantly higher in NAFLD patients than in subjects without NAFLD (183.6 ± 81.4 vs 86.7 ± 56.8 pmol/L, p<0.001; figure 1A) and directly correlated with the diagnosis of NASH, severity of steatosis, intrahepatocyte ballooning and, subsequently, higher NAS score and SAF score, as shown in Table 2. In particular, pro-NT levels were significantly higher throughout increasing NAS score severity subgroups (p<0.001, Figure 1B) and this association persisted strongly significant after correcting for sex and age at the partial correlation analysis (r= 0.62, p< 0.001). Among clinical parameters, greater pro-NT levels correlated with age, female gender, T2D and parameters associated with insulin-resistance and impaired glucose metabolism, as detailed in table 2. No association was found, instead, with BMI and waist circumference.

The presence of biopsy-proven NAFLD was associated with female sex (r= 0.31, p= 0.02), higher pro-NT (r= 0.56, p<0.001), ALT (r= 0.30, p= 0.03) and lower AST/ALT (r= -0.37, p= 0.009). At the multivariate logistic regression analysis, higher pro-NT levels were associated with biopsy-proven NAFLD independently from possible confounders (Table 3). Higher pro-NT concentration predicts the presence of NAFLD with AUROC= 0.836 (95%C.I.: 0.73- 0.94, p<0.001, figure 2).

Pro-NT and US-detected NAFLD

Out of the 260 consecutive individuals undergoing metabolic characterization and liver US, 60% (n= 157) had a diagnosis of NAFLD; subjects with NAFLD had significantly higher plasma pro-NT levels than non-NAFLD individuals (190.78 ± 116.6 *vs* 154.3 ± 88.9 pmol/L, p= 0.003). Clinical and metabolic characteristics of this study cohort, according to the presence of NAFLD, are shown as Supplementary Data S1. At the bivariate analyses, greater pro-NT levels correlated with the presence of NAFLD (r= 0.19, p= 0.002), T2D (r= 0.25, p= 0.001) and female gender (r= 0.15, p= 0.05), whereas a trend towards positive association - not reaching statistical significance- was observed between higher pro-NT levels and the number of MS components (r= 0.11, p= 0.08). No association was found between pro-NT, age and indexes of body adiposity, such as BMI and waist circumference (Supplementary

Data S2). The multivariate logistic regression analysis confirmed that higher pro-NT correlated with the presence of NAFLD independently from age, gender, presence of T2D and number of MS components (Supplementary Data S3).

Discussion

This study demonstrates the existence of an association between circulating pro-NT levels and the presence and severity of biopsy-proven NAFLD and NASH in obese adults. The relationship between higher pro-NT and NAFLD was confirmed in a larger population of adults with diagnosis of fatty liver made with US examination but without signs of severe liver damage, thus reinforcing the evidence obtained in patients evaluated with liver histology.

Recently Li J et al. (29), in an extensive investigation on mechanisms behind the association between higher pro-NT and the development of obesity and cardio-metabolic diseases (30, 31), found significantly reduced intestinal fat absorption in NT-deficient mice and protection towards high fat diet-induced obesity, hepatic steatosis and insulin-resistance in comparison with the wild-type (29). As NT-deficient mice (29), NTR3-deficient mice are protected from high fat diet-induced obesity and fatty liver (41), indicating that NT-induced hepatic fat accumulation is mediated by both NTR1 and NTR3. In our study, higher pro-NT correlated with T2D and signatures of impaired glucose metabolism and insulin-resistance, but not with adiposity *per se*, in line with previous reports (30, 31). NAFLD represents an established cardiovascular risk factor (16) and may determine and worsen insulin-resistance, systemic inflammation (10) and metabolic complications of obesity (11, 12). Indeed, we observed a linear association between pro-NT, hepatic damage in NASH and parameters related to glucose metabolism impairment.

A possible weakness of this novel observation can be represented by the limited sample size of the cohort undergoing liver biopsy. However, obtaining samples for liver histology implies the use of invasive procedures, reasonably representing *per se* a limiting factor for study enrolment.

On the other hand, all the study participants underwent accurate metabolic characterization; the study was monocentric and all the procedures were performed by the same operator, strengthening the study design and the reliability of our results. Finally, the findings obtained in the main study population have been confirmed in an additional cohort undergoing hepatic US and metabolic phenotyping, reinforcing our results and making them applicable also in individuals with different degree of body adiposity and non-clinically relevant hepatic damage. Indeed, the association between plasma pro-NT and different measurements of NAFLD broadens the clinical utility of our findings.

Despite the cross-sectional design of our study does not allow to establish a causal nexus between these findings, it is plausible to hypothesize that increased pro-NT levels facilitate the absorption of fatty acids from small intestine promoting fat accumulation in specific sites, such as the liver. Thus, NT may lead to NAFLD/NASH in a dose-dependent manner and may act both directly and indirectly –through hepatic fat accumulation- in worsening insulin resistance and metabolic profile. Gut hormones regulation is currently considered as an appealing target for anti-obesity treatment (42, 43); in this context, our findings are intriguing and may put the basis for further investigation on novel therapeutic approaches to NAFLD. Moreover, pro-NT may represent a novel biomarker of NAFLD in individuals with and without obesity, with relevant implications in clinical practice. In conclusion, our study demonstrates for the first time that pro-NT levels predict the presence of biopsy-proven NAFLD in obese subjects and are associated with insulin resistance and detrimental metabolic profile. Studies on larger cohorts and longitudinal design are warranted to

investigate the possible role of NT in the development, progression and prognosis of NAFLD and NASH.

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Authors contribution. IB, OL, MOM and MGC conceived the study; IB, MGC, FL, DC and FAC coordinated the study, oversaw patient recruitment and finalized the dataset; FAC, IB and DC oversaw collection and analysis of biological samples; MOM and OM performed the experiments; IB, MCG and OM performed the statistical analyses; GS performed all the liver biopsies; CDC read all the biopsies and finalized the dataset; IB and MGC drafted the paper, which was reviewed by all authors. All authors read and approved the final manuscript.

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Duality of Interest.
The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Figure 1. Plasma pro-NT levels according to the presence of NAFLD (A) and NAS score (B). *T-Student's test, §ANOVA test; Bonferroni comparison between subgroups: NAS ≤ 2 vs #3-4, ^vs ≥ 5; NAS 3-4 vs °≥ 5.

Figure 2. Pro-NT AUROC for NAFLD.

Table 1. Clinical and biochemical characteristic of patients with and without biopsy-proven NAFLD. Student's T test. *Chi-square test. § Student's t-test. P values refer to the age-, gender-and BMI-adjusted partial correlation analyses, unless differently specified.

	no NAFLD (n=28)	NAFLD (n=32)	Partial correlation coefficient (adjusted for age, gender, BMI)	P-value
Age (years)	40.5 ± 12	43.2 ± 9.4	-	n.s.§
Gender (M%)	61.5%	32%	-	0.03*
BMI (Kg/m^2)	43.5±6.3	41.8±4.3	-	n.s.§
Waist circumference (cm)	129.4±16.9	128±7.7	-0.31	0.20
SBP (mmHg)	133±8.4	124.4±7.7	-0.26	0.17
DBP (mmHg)	85±8.7	85.9±22.1	-0.07	0.72
Total Cholesterol (mg/dl)	213±140.4	171±126	-0.32	0.07
HDL-C (mg/dl)	52±8.8	46.7±10.2	-0.05	0.77
LDL-C (mg/dl)	141.9±26.6	121.1±22.3	-0.40	0.03
Triglycerides (mg/dl)	118±70.3	134±43.1	0.34	0.056
FBG (mg/dl)	102.1±45.3	102.5±17.3	0.41	0.017
HbA1c (%/mmol/mol)	5.2±0.25	5.5±0.48	0.33	0.07
AST (IU/l)	22.1±7.9	24.1±10.4	0.14	0.44
ALT (IU/l)	25.3±17.2	32.7±15.6	0.37	0.03
AST/ALT	0.99±0.3	0.78±0.2	-0.45	0.008
FBI (µU/l)	20.3±13.2	16±11.2	0.24	0.23
HOMA-IR	3.6±3.1	4.1±3.2	0.29	0.14
HOMA-β%	123±140.4	171±126	0.09	0.67
T2D (%)	11%	13%	0.23	0.19
Pro-NT (pmol/L)	**86.7±56.8**	**183.6±81.4**	**0.35**	**<0.001§; 0.039**

Table 2. Pro-NT- Bivariate correlation analyses (Pearson's coefficient, *Spearman's coefficient, pro-NT is considered as a continue variable)

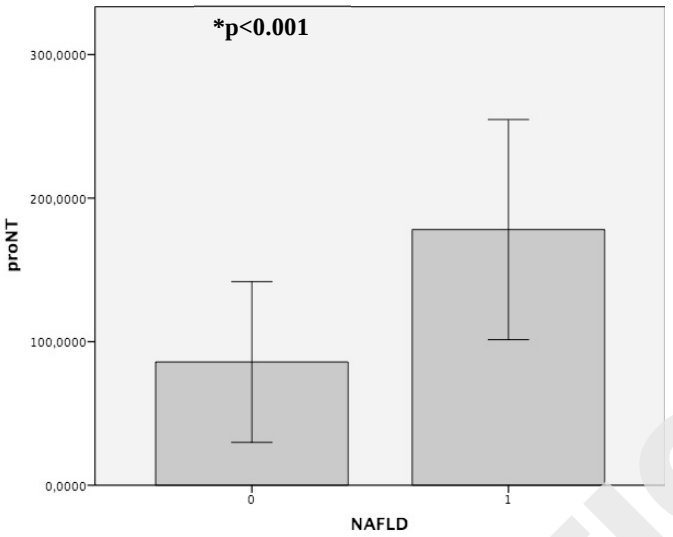
	Correlation coefficient	*p-value*
Age	**0.41**	**0.002**
Gender (M/F)	**0.34**	**0.005***
BMI	0.03	0.85
Waist circumference	-0.15	0.49
FBG	0.17	0.19
FBI	**0.54**	**0.002**
HbA1c	**0.62**	**<0.001**
Total Cholesterol	-0.20	0.17
HDL	-0.09	0.54
LDL	-0.28	0.065
Triglycerides	**0.30**	**0.05**
AST	0.02	0.86
ALT	0.09	0.56
Serum creatinine	**0.37**	**0.005**
HOMA-B	**0.69**	**0.003**
HOMA-IR	**0.47**	**0.009**
NAFLD yes/no	**0.59**	**<0.001***
SAF score	**0.36**	**0.02***
NAS: steatosis	**0.34**	**0.037***
NAS: intrahepatocyte ballooning	**0.54**	**<0.001***
NAS score	**0.46**	**0.003***
T2D yes/no	**0.31**	**0.02***

Table 3. Multivariate logistic regression analysis. The presence of NAFLD is the dependent variable. Pro-NT is considered as a continuous variable.

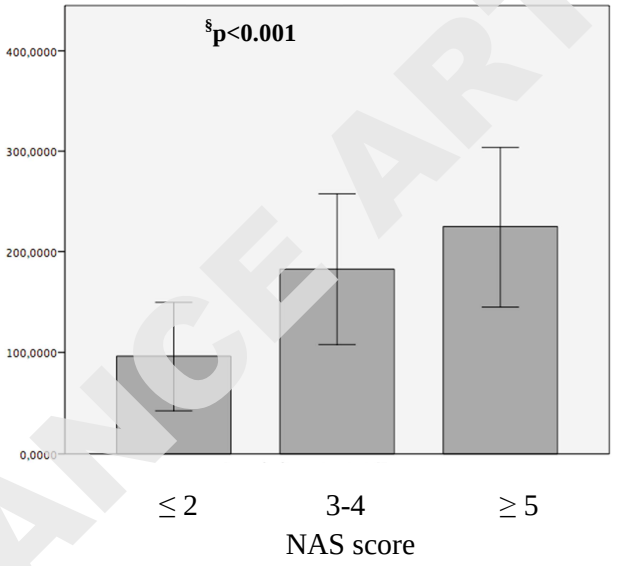
	B	*E.S.*	*Wald*	*P value*	*Odd Ratio*	*95% C.I.*	
						Inferior	*Superior*
Age	-0.027	0.043	0.378	0.54	0.97	0.89	1.06
Gender (M/F)	-2.146	1.281	2.807	0.09	0.12	0.01	1.44
AST/ALT	-2.527	1.873	1.819	0.18	0.08	0.002	3.14
ALT (IU/L)	-0.010	0.032	0.106	0.74	0.99	0.93	1.05
Pro-NT (pmol/L)	**0.022**	**0.010**	**5.094**	**0.02**	**1.02**	**1.003**	**1.04**
Constant	2.792	3.087	0.818	0.37	16.31		

Cox and Snell R^2= 0.412

A.



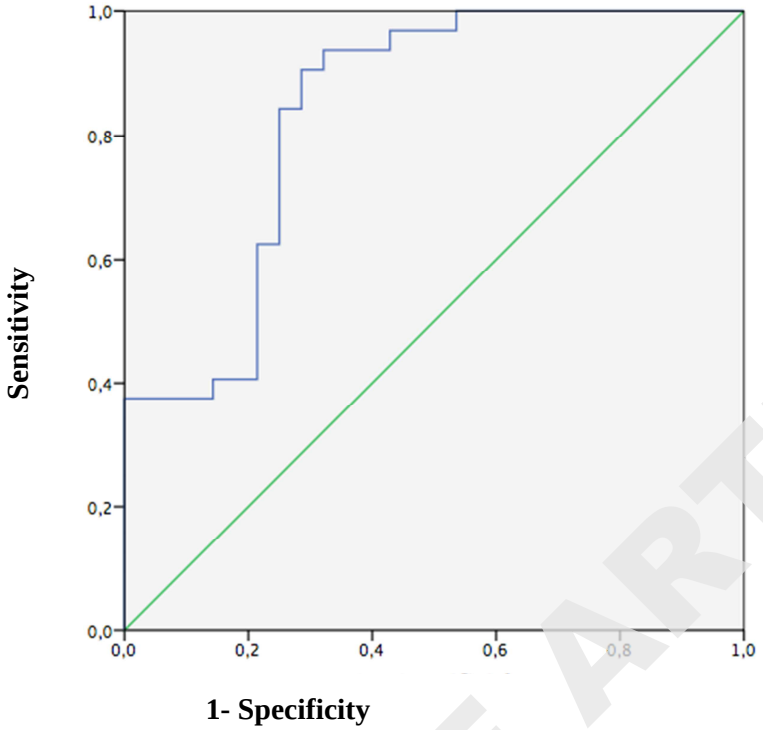
B.



NAS score	n. of subjects	Pro-NT (pmol/L)	P value
≤ 2	37	92.3 ± 53.8	#0.001; ^<0.001
3-4	11	182.8 ± 75	#0.001; °0.39
≥ 5	12	224.6 ± 79.1	^<0.001; °0.39

Figure 1. Plasma pro-NT levels according to the presence of NAFLD (A) and NAS score (B). *T-Student's test, §ANOVA test; Bonferroni comparison between subgroups: NAS ≤ 2 vs #3-4, ^vs ≥ 5; NAS 3-4 vs °≥ 5.

ROC CURVE



Area under the curve

Area	Standard error[a]	Asintotic significance[b]	Asintotic 95%Confidence Interval	
			Lower limit	Upper limit
0.836	**0.054**	**<0.001**	**0.731**	**0.941**

a. Based on non-parametric assumption

b. Null hypothesis: real area = 0.5

Sensitivity: 84% - Specificity: 75% per plasma pro-NT values > 107 pg/ml

Figure 2. Pro-NT AUROC for NAFLD.