UNIMORE
UNIVERSITÀ DEGLI STUDI DI MODENA E REGGIO EMILIA

UNIVERSITÀ DEGLI STUDI DI MODENA E REGGIO EMILIA

Dottorato di ricerca in
Clinical and experimental medicine (CEM)
- Medicina clinica e sperimentale -

Ciclo XXXVIII

DIABETIC NEUROPATHY AND LIVER FIBROSIS IN TYPE 2 DIABETES MELLITUS: AN OBSERVATIONAL CROSS-SECTIONAL ANALYSIS OF DATE FROM CLINICAL PRACTICE

Candidato **Carla Greco**

Relatore (Tutor): **Prof.ssa Manuela Simoni**

Coordinatore del Corso di Dottorato: **Prof. Marco Vinceti**

TABLE OF CONTENTS

ABSTRACT

BACKGROUND. Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) and Diabetic Peripheral Neuropathy (DPN) share etiological aspects and clinical correlates. On the other hand, the relationship between liver fibrosis and DPN has not been well investigated, so far.

OBJECTIVES. We aimed to evaluate the association between liver fibrosis and DPN and to investigate the liver non-invasive tests (NITs) of fibrosis as predictors of DPN.

METHODS. We conducted an observational, cross-sectional, retrospective study including individuals with type 2 diabetes mellitus (T2DM) and hepatic steatosis that performed an evaluation of DPN. Neurological assessment included evaluation of neuropathic symptoms and deficits using validated scoring systems, i.e. Questionnaire of the Michigan Neuropathy Screening Instrument (MNSI-Q), Michigan Diabetic Neuropathy Score (MDNS) and Diabetic Neuropathy Index (DNI). The definition of DPN required the presence of at least one abnormality among neuropathic symptoms and signs. Risk of advanced fibrosis was estimated using the Fibrosis-4 (FIB-4) score, the NAFLD Fibrosis Score (NFS), the AST/ALT ratio, and the AST to platelet ratio index (APRI). Finally, the presence of fibrosis has been investigated using the vibration controlled transient elastography (VCTE, Fibroscan®). Results are expressed in liver stiffness measurement (LSM) as kiloPascals (kPa) and LSM value ≥7.0 kPa defined the presence of significant fibrosis.

RESULTS. Eighty-six T2DM subjects (mean age 59.22 ± 13.18 years, duration diabetes 9.61 ± 8.87 years, glycated haemoglobin (HbA1c) 57.71 ± 14.28 mmol/mol, 61 males –

70.9%) were included. DPN was detected in 43% of the cohort. Individuals with DPN were older (p<0.001), with longer diabetes duration (p=0.009), and characterized by higher prevalence of increased urinary albumin excretion (p=0.011), chronic kidney disease (p=0.038) and retinopathy (p=0.021). Significantly higher risk scores of liver fibrosis (FIB-4 and AST/ALT) in subjects with clinical diagnosis of DPN compared to those without DPN have been detected (FIB-4: 1.23 ± 0.66 *vs* 1.63 ± 0.85; p 0.018; AST/ALT: 0.89 ± 0.23 *vs* 1.11 ± 0.61; p 0.026). Moreover, DPN group showed higher value of LSM (6.56 ± 4.23 kPa *vs* 7.12 ± 3.58 kPa; p 0.619) and MDNS score was significantly directly related to LSM (Rho: 0.304, p=0.026). Considering neurological examination, higher prevalence of alteration in vibration sensation by diapason or in tendon reflexes evaluation was observed in subjects with documented fibrosis compared to LSM < 7kPa (p 0.025 and p 0.042, respectively). Finally, the qualitative evaluation of vibration sensation or tendon reflexes in individual at high risk of liver fibrosis at FIB-4 and AST/ALT indices was significantly associated with DPN (FIB-4 + abnormal vibration: p 0.047; FIB-4 + abnormal reflex: p 0.013; AST/ALT + abnormal vibration: p <0.001; AST/ALT + abnormal reflex: p <0.001).

CONCLUSIONS. Exploring easy-to-apply and widely screening methods, the simple qualitative evaluation of vibration perception or tendon reflexes would be useful and effective in identifying DPN in T2DM with MASLD at high risk of fibrotic evolution.

1.INTRODUCTION

1.1. DIABETIC PERIPHERAL NEUROPATHY (DPN)

Diabetic peripheral neuropathy (DPN) is a diabetes complication that leads to the damage of small and large nerve fibers (1). DPN occurs in at least 20% of subjects with type 1 diabetes mellitus (T1DM) after 20 years of disease, and in 10-15% of subjects with a new diagnosis of type 2 diabetes mellitus (T2DM), up to 50% in subjects with T2DM after 10 years of disease (2). The pathogenesis is multifactorial and complex; however, in a simplistic way, oxidative and inflammatory stress can cause nerve cell injury, in the context of metabolic disfunction (2). Usually, the early symptoms are pain and dysesthesia due to the involvement of the small fibers, while the involvement of the large ones can lead to loss of protective sensation and numbness (1). The diagnosis is a diagnosis of exclusion of other neuropathies' causes, and it is based on the evaluation of neuropathic symptoms and deficits using validated scoring systems. For the evaluation of neuropathic symptoms, the majority of clinicians uses the Questionnaire of the Michigan Neuropathy Screening Instrument (MNSI-Q); on the other hand, for the evaluation of neuropathic signs, the Michigan Diabetic Neuropathy Score (MDNS) and the Diabetic Neuropathy Index (DNI) are the most used scores. To detect small fibers' deficits the tests that can be used are pinprick and temperature sensation, to detect large fibers' deficits the tests that can be used are lower-extremity reflexes, vibration perception and 10-g monofilament, and finally to detect protective sensation the test that is possible to use is the 10-g monofilament (1, 3, 4). The definition of DPN required the presence of at least one abnormality among neuropathic symptoms or signs (5).

There is no a specific pharmacological therapy to treat DPN; it is mandatory to optimize glycaemic control, blood pressure and lipid control to slow the progression of it (1). For neuropathic pain, gabapentin, serotonin-norepinephrine reuptake inhibitors, tricyclic antidepressant, and sodium channel blockers are recommended (1).

1.2. METABOLIC DYSFUNCTION-ASSOCIATED STEATOTIC LIVER DISEASE (MASLD) AND ADVANCED FORMS OF LIVER DAMAGE

Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) means the presence of hepatic steatosis in conjunction with one cardiometabolic risk factor (6), and encompasses a broad spectrum of liver disease, from hepatic steatosis to cirrhosis, including liver fibrosis (7). This term replaced in June 2023 the known term “Non-Alcoholic Fatty Liver Disease” (NAFLD), thanks to a multi-society Delphi consensus statement (6). Anyway, 99% of subjects with NAFLD meet MASLD criteria, but the new term reflects better the pathophysiology and the cardiometabolic implications (8). Nowadays, 30%-38% of the entire population has MASLD, and its prevalence is increasing alongside the prevalence of obesity and obesity-related diseases (9-11). Obesity, T2DM and insulin resistance are recognized as factors underlying the development of MASLD (12).

The criteria for the diagnosis of MASLD include the presence of hepatic steatosis (detected by imaging or biopsy) plus the presence of one between these five features:

1) body mass index (BMI) ≥ 25 kg/m^2(≥ 23 kg/m^2 in Asian) or waist circumference > 94 cm in men, > 80 cm in women, or ethnicity adjusted;

2) fasting serum glucose ≥ 100 mg/dL (≥ 5.6 mmol/L) or 2-hour post-load glucose level ≥ 140 mg/dL (≥ 7.8 mmol/L) or glycated haemoglobin (HbA1c) ≥ 5.7% or specific drug treatment;

3) blood pressure ≥ 130/85 mmHg or specific drug treatment;

4) plasma triglycerides ≥ 150 mg/dL (≥ 1.70 mmol/L) or specific drug treatment;

5) plasma high-density lipoprotein (HDL) cholesterol < 40 mg/dL (< 1.0 mmol/L) for men and < 50 mg/dL (< 1.3 mmol/L) for women or specific drug treatment (9).

The term “Metabolic dysfunction-associated steatohepatitis (MASH)” means the more severe form of MASLD, that is recognized histologically by the presence of lobular inflammation and hepatocyte ballooning. Subjects with MASH are at a greater risk of fibrosis progression (9, 13). In turn, MASH can progress to more advanced degrees of liver disease such as fibrosis, cirrhosis and even hepatocellular carcinoma (HCC). Fibrosis is scarring of the liver caused by inflammation and chronic cellular damage.

Cardiovascular disease (CVD) is the leading cause of mortality in subjects with MASLD (9); otherwise, the presence of advanced liver fibrosis can lead to an increase of liver-related events, such as gastroesophageal varices, variceal hemorrhage, ascites, hepatocellular carcinoma (HCC) (14), and death (15).

Hyperglycemia, insulin resistance and lipotoxicity are the main determinants of liver damage (16-18); further, the lipotoxic environment leads the activation of liver macrophages that can worsen the hepatocellular injury (15).

Taking into account these considerations it is clear that MASLD and in particular the liver fibrosis are associated with metabolic diseases and overweight-related diseases, in particular with obesity, hypertension, dyslipidemia, obstructive sleep apnea, CVD, chronic kidney disease (CKD), and T2DM. Categories at risk of advanced fibrosis, like T2DM, obesity, moderate alcohol users, first-degree relatives of subjects with cirrhosis due to MASLD/MASH must undergo to a screening based on Fibrosis-4 (FIB-4) score (15). FIB-4 score is calculated using a simple algorithm based on age, aspartate transaminase (AST), alanine transaminase (ALT) and platelet count. Depending on FIB-4 score three fibrosis risk categories are identified: low risk (FIB-4 score < 1.3), intermediate risk (FIB-4 1.3-2.67), and high risk (FIB-4 > 2.67). Vibration-controlled elastography (VCTE) (Fibroscan®), one of the most commonly used methods to evaluate liver stiffness, is indicated when FIB-4 is ≥ 1.3 (15).

1.3 ASSOCIATION BETWEEN METABOLIC DYSFUNCTION-ASSOCIATED STEATOTIC LIVER DISEASE AND TYPE 2 DIABETES MELLITUS

As can be understood from the definition, the association between MASLD and cardiovascular risk factors, including T2DM, is clear; indeed, MASLD and T2DM share common pathological aspects, the most relevant of which is insulin resistance and its

related metabolic dysfunction (20). In a recent study, the prevalence of MASLD in subjects with T2DM was 64%, while the prevalence of significant fibrosis (defined as liver stiffness measurement (LSM) ≥ 8.1 kiloPascals (kPa)), advanced fibrosis (defined as LSM ≥12.1 kPa), and cirrhosis due to MASLD were 17%, 11%, and 3%, respectively (7). Another meta-analysis found that subjects with T2DM and MASH are younger, and with a higher BMI; this suggests that the course could be more progressive, and therefore these subjects should receive more aggressive management strategies for the risk of CVD and liver-related events (21). Indeed, T2DM is a recognized independent risk factor for MASH and for advanced liver fibrosis (9, 22), and another meta-analysis found out that MASLD is associated with an increased risk of all-cause mortality among the T2DM population (23). On the other hand, Mantovani and colleagues in their meta-analysis found that MASLD doubles the risk of developing diabetes, irrespective of age, sex, adiposity measures and other metabolic risk factors (22). It seems that the relationship between MASLD and T2DM is bidirectional: MASLD leads to a reduction in insulin sensitivity, while hyperglycemia seems to promote hepatocellular injury and liver fibrosis (15). On the other hand, this coexistence contributes also to the higher risk of T2DM-related chronic vascular complications (24).

1.3.1 Metabolic Dysfunction-Associated Steatotic Liver Disease and macrovascular complication in type 2 diabetes mellitus

Nowadays, MASLD is a recognized risk factor for major adverse cardiovascular events and for CVD morbidity and mortality, as many cohort studies and meta-analyses proved.

There is a comprehensive recent review that summarized these studies (20); in the current paper we limit ourselves to mentioning a recent and large meta-analysis of 36 longitudinal studies (25). The authors demonstrated in 5.802.226 middle-aged individuals (mean age 53 years [standard deviation (SD) 7]) a moderately increased risk of fatal or non-fatal CVD events (pooled random-effects Hazard ratio (HR) 1.45, 95% confidence interval (CI) 1.31-1.61; heterogeneity (I2)=86.18%), that increase across the severity of MASLD, especially in the stage of fibrosis (pooled random-effects HR 2.50, 95% CI 1.68-3.72; I2=73.84%) (25).

1.3.2 Metabolic Dysfunction-Associated Steatotic Liver Disease and microvascular complication in type 2 diabetes mellitus

In contrast to the associations of MASLD and macrovascular diseases, the effect of MASLD on the development of microvascular complications in individuals with T2DM is less frequently investigated. A meta-analysis of 9 observational longitudinal studies involving non-hospitalized subjects with T2DM (96.595 adult individuals, 34.1% with MASLD) reported an increased risk of incident CKD in individuals with MASLD (HR: 1.37) (26). Moreover, a recent prospective cohort study found an association between MASLD and nephropathy and neuropathy in subject with T2DM (27), and a retrospective study found that MASLD increases the risk of end-stage renal disease in subjects with T2DM and diabetic nephropathy (28). Anyway, the data remains conflicting, since in other studies this association was not found (29). Similarly, MASLD was found to be associated with an increased prevalence of proliferative/laser-treated retinopathy in T2DM individuals in the cross-sectional evaluation from Valpolicella Study (30), even if in other

studies this association was not proven (29). Considering DPN, a significantly increased DPN prevalence among T2DM subjects with MASLD was observed in a meta-analysis including thirteen cross-sectional studies (9614 participants) (31). Recently, using the outpatient part of the nationwide Swedish Patient Register, Ebert *et al* have collected individuals with a first MASLD diagnosis (number (N) = 6785) and matched these with up to 10 reference individuals from the general population (N = 61136) (32). The authors demonstrated in a median follow up of 5.7 years an incidence rate of microvascular diseases > twofold higher in subjects with MASLD (10.8 per 1000 person-years [95% CI = 9.9– 11.8]) versus reference individuals (4.7 per 1000 person-years [95%CI = 4.5–4.9]) (32). When analysed separately, MASLD was related to a 46% and 38% increased of CKD and retinopathy, respectively (32). For neuropathy, very few outcomes were found; thus, the regressions are not considered reliable and so the results inconclusive (32).

1.4 LIVER FIBROSIS AND VASCULAR COMPLICATION IN TYPE 2 DIABETES MELLITUS

The associations between liver fibrosis and the presence of macrovascular and microvascular complications have been reported more recently. As we wrote before, fibrosis is associated to an increased risk of fatal or non-fatal CVD events (25). Moreover, a multicenter study on 442 subjects with T2DM demonstrated that significant liver fibrosis is associated with an increased likelihood of having myocardial infarction (adjusted-odds ratio (OR) 6.61, 95%CI 1.66-37.4), peripheral polyneuropathy (adjusted-OR 4.55, 95%CI 1.25-16.6), CKD (adjusted-OR 4.54, 95%CI 1.24-16.6), or retinopathy

(adjusted-OR 1.81, 95%CI 1.62-1.97), independently of cardiometabolic risk factors, diabetes-related variables, and other potential confounders (33), suggesting a relationship between liver fibrosis and macro- and microvascular complications of T2DM. Finally, an Italian study demonstrated that liver fibrosis is associated with macrovascular and microvascular complications (in particular CKD and retinopathy) (34).

1.4.1 Association between liver fibrosis and diabetic peripheral neuropathy

Focusing on DPN, pathogenetic pathways implicated in DPN share with metabolic liver disease risk factors and etiological aspects including hyperglycaemia, insulin resistance and dyslipidaemia. Further, liver fibrosis *per se* might also be involved in DPN pathogenesis. In details, bile acid, synthesized from cholesterol in the liver, might function as a signalling molecule through a variety of receptors to regulate their own synthesis and other metabolic processes, such as glucose, lipid and energy homeostasis, and was implicated in the occurrence of liver fibrosis (35). TGR5 (Takeda G-protein-coupled receptor 5), one of the receptors of bile acid, is widely distributed and expressed in neurons (35), and might mediate its role in the pathogenesis of DPN through inflammation. Indeed, TGR5 plays a key role in different cellular functions by affecting multiple pathways including extracellular signal-regulated kinase (ERK), nuclear factor kB (NF-kB), signal transducer and activator of transcription 3 (STAT3), and Akt (36, 37). Indeed, interacting with TGR5, bile acid would inhibit NF-kB activation, leading to an anti-inflammatory effect (35). Besides, TGR5 activation stimulates cyclic adenosine monophosphate (cAMP)-related pathways (38, 39), resulting as promising agent for

inducing myelination in the development of peripheral nerves or remyelination (40). In an experimental model in vivo, treatment with TGR5 agonist markedly improved nerve regeneration and functional recovery in sciatic nerve crush-injured mice (41).

Finally, several practical liver non-invasive tests (NITs) have been developed and validated to detect or exclude advanced liver fibrosis. Among these, the NAFLD fibrosis score (NFS) and the FIB-4 are the most commonly used scoring systems, and they have been endorsed by international guidelines (12, 42, 43). The studies conducted so far have demonstrated that NFS and FIB-4 scores are able to predict mortality (44-47), cardiovascular events, CKD and liver-related complications in MASLD individuals (46, 47). Focusing on DPN, there are few studies that have evaluated the association with these NITs showing inconclusive findings (27, 48, 49).

2. OBJECTIVE OF THE STUDY

Therefore, the association between DPN and liver fibrosis is an emerging finding while the association between NITs and DPN still has inconclusive evidence. Since results of the previous studies are controversial, the present study aimed to investigate the association of liver fibrosis and DPN considering NITs as non-invasive fibrosis scoring systems for the screening and the evaluation of stiffness to diagnose liver fibrosis.

3. MATERIALS AND METHODS

An observational, cross-sectional, retrospective study was conducted enrolling subjects with T2DM and hepatic steatosis, underwent to neurological evaluation at the University Hospital of Modena (Italy) diabetes services between January 2022 and February 2024.

3.1. STUDY POPULATION

Subjects with T2DM and hepatic steatosis that performed an evaluation of DPN were enrolled at the University Hospital of Modena (Italy) diabetes services between January 2022 and February 2024.

Inclusion criteria: previous diagnosis of T2DM according to American Diabetes Association (ADA) guidelines (50), presence of hepatic steatosis, age ≥ 18 years, previous evaluation of DPN.

There were not specific exclusion criteria.

3.2. STUDY DESIGN

We collected data on previous biochemical assessment, evaluation of DPN, and evaluation of liver fibrosis. The maximum length time between biochemical assessment, evaluation of DPN and evaluation of liver fibrosis was three months. Demographic (age and sex), anthropometric (height, weight, waist and hip circumference), clinical (smoking, physical activity, diabetes duration, macro- and microvascular complication of T2DM, comorbidities) and pharmacological data were collected.

3.2.1. Biochemical assessment

In this study we collected the following biochemical parameters: fasting glucose (mg/dl), HbA1c (mmol/mol), AST (U/L), ALT (U/L), gamma-glutamyl transferase (gGT) (U/L), albumin (g/dl), total cholesterol (mg/dl), HDL (mg/dl), low-density lipoprotein (LDL) (mg/dl), triglycerides (mg/dl), creatinine (mg/dl), estimated glomerular filtration rate (eGFR) (ml/min), hemochrome.

3.2.2. Evaluation of diabetic peripheral neuropathy

Neurological assessment included evaluation of neuropathic symptoms and deficits using validated scoring systems, i.e. the MNSI-Q, the MDNS and the DNI. MNSI-Q, MDND, DNI are defined according to standardized scores (3, 4). The definition of DPN required the presence of at least one abnormality among neuropathic symptoms or signs (5).

3.2.3. Evaluation of liver steatosis

The presence of hepatic steatosis was detected by liver ultrasonography (US). The presence and severity of hepatic steatosis by US is defined according to characteristic imaging features, namely bright liver pattern, liver-kidney contrast, vascular blurring and/or deep hepatic attenuation.

3.2.4. Evaluation of liver fibrosis (NITs)

Risk of advanced fibrosis is estimated using the FIB-4 score (51), the NFS (52), the AST/ALT ratio, and the AST to platelet ratio index (APRI) (53). Finally, the presence of fibrosis is investigated using VCTE (Fibroscan®). Two Fibroscan® probes are available: the M probe and the XL probe; the latter has been designed specifically for use in subjects with obesity. LSM were performed after at least 3 h of fasting, by two highly experienced operators (> 500 exams). Measurements were conducted on the right lobe of the liver in an area without focal lesions. Results, automatically generated by the software, are expressed in LSM as kPa. The LSM were considered reliable only if 10 successful measurements were obtained corresponding to the median of 10 valid measurements. According to literature consensus, LSM value ≥7.0 kPa (54) defined the presence of significant liver fibrosis (≥F2).

3.3. ETHICS

This study was performed in accordance with the Declaration of Helsinki. Source documents of research material in form of paper files and electronic files, and all data sets are deposited at the Unit of Endocrinology of Azienda Ospedaliero-Universitaria of Modena (Italy) and are available upon request. The Institutional Review Board of Modena and the Ethical Committee of Modena approved this study (code 0022400/21, approved on July 21, 2021). According to the Ethics Committee recommendations and considering the retrospective study design, informed consent was waived since data were collected anonymously.

3.4. STATISTICAL ANALYSIS

Continuous and categorical data were compared performing Mann Whitney test and Fisher exact test. Bivariate correlation analyses were performed by Rho's Spearman combining DPN scores, risk indices for liver steatosis/fibrosis and those variables that changed between subjects with or without DPN. Values are reported as mean ± SD. Statistical analysis was performed using the "Statistical Package for the Social Sciences" software for Windows (version 27.0; SPSS Inc., Chicago, IL, USA). For all comparisons, $p < 0.050$ was considered statistically significant.

4. RESULTS

4.1. CLINICAL CHARACTERISTICS OF THE STUDY POPULATION

A total of 86 individuals with T2DM and hepatic steatosis were enrolled in the present study, including 61 men (70.9%) and 25 women (29.1%). The mean age at baseline was 59.22 ± 13.18 years, ranging from 21 to 85 years. All basic clinical characteristics, including age, BMI, duration of T2DM, glycaemic control, presence of diabetes complications, blood lipid, kidney, and liver function and kidney function indicators, therapy, etc, are presented in **Table 1a** and in **Table 1b**.

Table 1a. Clinical characteristics of 86 subjects with type 2 diabetes mellitus and hepatic steatosis.

		Overall (n=86)
General characteristics		
	Male (%)	61 (70.9)
	Age (years)	59.22 ± 13.18 (21-85)
	Duration of diabetes (years)	9.61 ± 8.87 (1-42)
	Body weight (Kg)	93.01 ± 23.69 (48.5-173)
	BMI (Kg/m^2)	32.60 ± 7.45 (18-53)
	With obesity (%)	52 (60.5)
	Waist circumference (cm)	111.24 ± 15.88 (65-145)
	HbA1c (mmol/mol)	57.71 ± 14.28 (33-95)
Complications and comorbidities		
	With diabetic retinopathy (%)	7/79 (8.9)
	With DPN (%)	37 (43)
	With albuminuria (%)	24/83 (28.9)
	With chronic kidney disease – low eGFR (%)	13 (15.1)
	With hypertension (%)	69 (80.2)
	With dyslipidemia (%)	78 (90.7)
	With ischemic heart disease (%)	28 (32.6)
	With heart failure (%)	11 (12.8)

	With cerebrovascular disease (%)	30 (34.9)
	With peripheral vascular disease (%)	8/54 (14.8)

[Footnotes: BMI, body mass index; DPN, diabetic peripheral neuropathy; estimated glomerular filtration rate, eGFR; HbA1c, glycated haemoglobin].

Table 1b. General, biochemical and therapeutical information of 86 subjects with type 2 diabetes mellitus and hepatic steatosis.

		Overall (n=86)
General characteristics		
	Hip circumference (cm)	112.49 ± 15.64 (85-155)
	Previous or current smoking (%)	60 (69.80)
	Physical activity (%)	16 (18.6)
Biochemical evaluations		
	Fasting glucose (mg/dl)	147.86 ± 44.72 (73-306)
	AST (U/L)	29.17 ± 20.01 (12-128)
	ALT (U/L)	32.02 ± 20.78 (8-132)
	γ-GT (U/L)	48.75 ± 53.07 (8-345)
	Albumin (g/dl)	4.13 ± 0.32 (3.29-4.80)
	Total cholesterol (mg/dl)	156.04 ± 46.91 (76-270)
	HDL (mg/dl)	44.92 ± 11.28 (18-79)
	LDL (mg/dl)	92.26 ± 36.66 (33-191)
	Triglycerides (mg/dl)	156.39 ± 87.04 (49-507)
Therapy		
	With antihypertensive therapy (%)	67 (77.9)

	With antidyslipidemic therapy (%)	76 (88.4)
	With basal-bolus insulin therapy (%)	10 (11.6)
	With mixed antidiabetic therapy (%)	16 (18.6)
	With no-insulin antidiabetic therapy (%)	63 (73.3)
	Metformin (%)	69 (80.2)
	Pioglitazone (%)	0
	Acarbose (%)	0
	SU (%)	0
	DPPIVi (%)	6 (7)
	GLP1Ra (%)	46 (53.5)
	SGLT2i (%)	27 (31.4)

[Footnotes: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DPPIVi, dipeptidyl peptidase-4 inhibitor; γ-GT, gamma-glutamyl transferase; GLP1Ra, glucagon-like peptide-1 receptor agonist; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SGLT2i, sodium-glucose cotransporter 2 inhibitor; SU, sulfonylureas].

4.2. PREVALENCE AND CLINICAL CHARACTERISTICS OF INDIVIDUALS WITH DIABETIC PERIPHERAL NEUROPATHY

DPN was detected (DPN^+) in 43% of the overall cohort (37 subjects). DPN^+ subjects were older ($p<0.001$) and with longer diabetes duration ($p=0.009$). Moreover, DPN^+ individuals show a lower BMI ($p=0.034$), and a higher presence of diabetic complications, in particular retinopathy ($p=0.021$), impaired urinary albumin excretion ($p=0.011$), and chronic kidney disease ($p=0.038$) (**Table 2a**). No significant differences were also

observed in the current use of any antidiabetic drugs between subjects with and without DPN (**Table 2b**).

Table 2a. Clinical characteristics in individuals with and without diabetic peripheral neuropathy.

	no-DPN (n = 49)	DPN (n = 37)	p
General and anthropometric characteristics			
Male (%)	36 (73.5)	25 (67.6)	0.551
Age (years)	54.76 ± 13.04	65.14 ± 10.97	***< 0.001***
Duration of diabetes (years)	7.49 ± 7.60	12.5 ± 9.71	***0.009***
Body weight (Kg)	97.30 ± 25.12	87.17 ± 20.53	0.051
BMI (Kg/m²)	34.07 ± 8.08	30.62 ± 6.06	***0.034***
With obesity (%)	32 (65.3)	20 (54.1)	0.291
Waist circumference (cm)	112.86 ± 15.74	109.19 ± 16.08	0.341
Hip circumference (cm)	115.71 ±16.25	108.63 ± 14.20	0.062
Previous or current smoking (%)	37 (75.5)	23 (62.2)	0.182
Physical activity (%)	10 (20.4)	6 (16.2)	0.621
Biochemical evaluations			
Fasting glucose (mg/dl)	142.78 ± 41.22	154.59 ± 48.74	0.227
HbA1c (mmol/mol)	57.76 ± 15.55	57.65 ± 12.61	0.973
AST (U/L)	29.54 ± 19.37	28.67 ± 21.10	0.875
ALT (U/L)	34.94 ± 24.25	28.14 ± 14.41	0.139
γ-GT (U/L)	44.64 ± 39.32	54.53 ± 68.21	0.424
Total cholesterol (mg/dl)	148.27 ± 48.69	167.00 ± 42.58	0.075

	HDL (mg/dl)	43.08 ± 11.47	47.43 ± 10.66	0.083
	LDL (mg/dl)	88.34 ± 36.69	98.15 ± 36.40	0.244
	Triglycerides (mg/dl)	150.58 ± 73.70	164.34 ± 103.20	0.480
Complications and comorbidities				
	With diabetic retinopathy (%)	1 (2.3)	6 (17.1)	***0.021***
	With albuminuria (%)	9 (18.4)	15 (44.1)	***0.011***
	With chronic kidney disease – low eGFR (%)	4 (8.2)	9 (24.3)	***0.038***
	With hypertension (%)	36 (73.5)	33 (89.2)	0.070
	With dyslipidemia (%)	43 (87.8)	35 (94.6)	0.280
	With ischemic heart disease (%)	15 (30.6)	13 (35.1)	0.658
	With heart failure (%)	4 (8.2)	7 (18.9)	0.139
	With cerebrovascular disease (%)	15 (30.6)	15 (40.5)	0.339
	With peripheral vascular disease (%)	3 (10.3)	5 (20)	0.319

[Footnotes: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DPN, diabetic peripheral neuropathy; estimated glomerular filtration rate, eGFR; HbA1c, glycated haemoglobin; γ-GT, gamma-glutamyl transferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein].

Table 2b. Anti-diabetic treatment according to the clinical diagnosis of diabetic peripheral neuropathy.

	no-DPN (n = 49)	**DPN (n = 37)**	**p**
Therapy			
With antihypertensive therapy (%)	35 (71.4)	32 (86.5)	0.096
With antidyslipidemic therapy (%)	41 (83.7)	35 (94.6)	0.118
With no-insulin antidiabetic therapy (%)	38 (77.6)	25 (67.6)	0.300
With mixed antidiabetic therapy (%)	8 (16.3)	8 (21.6)	0.532

With basal-bolus insulin therapy (%)	5 (10.2)	5 (13.5)	0.635

[Footnotes: DPN, diabetic peripheral neuropathy].

4.3. ASSOCIATIONS BETWEEN FIBROSIS INDICES AND LIVER STIFFNESS MEASUREMENT, AND THE PRESENCE OF DIABETIC PERIPHERAL NEUROPATHY

Among NITs of fibrosis, FIB-4 and AST/ALT ratio scores were higher in DPN^+ *vs* DPN^- (p=0.018, and p=0.026, respectively). Finally, LSM by Fibroscan has been investigated in 54 subject (62.8%) demonstrating a prevalence of liver fibrosis of 25.9% in the evaluated cohort. Although fibrosis appeared more prevalent in DPN^+ *vs* DPN^- (40 versus 17.5%), the small sample size did not allow to reach statistical significance (p=0.073) (**Table 3**).

Table 3. Non-invasive biomarkers of liver fibrosis (86 subjects) and liver stiffness measurement (54 subjects) in whole population and according to the DPN diagnosis.

Non-invasive biomarkers of fibrosis	**(n = 86)**	**no-DPN (n = 49)**	**DPN (n = 37)**	**p**
FIB-4 score (mean ± SD)	1.41 ± 0.77	1.23 ± 0.66	1.63 ± 0.85	***0.018***
FIB-4 higher risk***** score (%)	32.5	12 (26.7)	14 (38.9)	0.242
NFS (mean ± SD)	- 0,09 ± 1.37	- 0,37 ± 1.43	0.19 ± 1.20	0.086
NFS higher risk***** score (%)	89.7	33 (86.8)	30 (93.8)	0.337
AST/ALT ratio (mean ± SD)	0.97 ± 0.43	0.89 ± 0.23	1.11 ± 0.61	***0.026***
AST/ALT ratio higher risk***** score (%)	72.3	34 (69.4)	29 (78.4)	0.473

APRI score (mean ± SD)	0.34 ± 0.26	0.33 ± 0.27	0.34 ± 0.22	0.935
APRI higher risk* score (%)	19	5 (10.9)	5 (13.5)	0.713
Instrumental evaluation of fibrosis	**(n = 54)**	**no-DPN (n =34)**	**DPN (n = 20)**	**p**
Mean LSM (kPa)	6.83 ± 4.04 (2.8-22)	6.56 ± 4.23	7.12 ± 3.58	0.619
With LSM ≥ 7 kPa (%)	25.9	17.5	40	0.073

****** intermediate and high risk*

[Footnotes: APRI, aspartate aminotransferase to platelet ratio index; AST/ALT, aspartate aminotransferase/alanine aminotransferase ratio; DPN, diabetic peripheral neuropathy; FIB-4, Fibrosis-4; kPa, kilopascal; LSM, liver stiffness measurement; NFS, NAFLD (non-alcoholic fatty liver disease) fibrosis score].

4.4. ASSOCIATIONS BETWEEN NITs (FIBROSIS INDICES AND LIVER STIFNESS MEASUREMENT) AND THE NEUROLOGICAL CLINICAL EVALUATION PERFORMED WITH MNSI-Q, DNI, MDNS

Then, we stratified the neurological clinical evaluation (performed with MNSI-Q, DNI, MDNS) according to FIB-4, AST/ALT ratio risk scores and LSM (**Table 4**). Subjects with LSM ≥7 kPa show higher MNSI-Q score (p=0.05), and MDNS score (p=0.006), and a more prevalence of MDNS abnormality (p=0.05), abnormal vibration sensation at great toe (p=0.025), and abnormal reflex test at upper and lower limbs (p=0.042). Moreover, we studied the prevalence of the combination between high risk of having liver fibrosis (evaluated by FIB-4 score and AST/ALT ratio) and one abnormal neurological examination according to the clinical diagnosis of DPN (**Table 5**). DPN^{+} individuals

showed higher risk FIB-4 score + abnormal vibration sensation (p=0.047), higher risk FIB-4 + abnormal reflex (p=0.013), higher risk AST/ALT ratio + abnormal vibration sensation (p<0.001), higher risk AST/ALT ratio + abnormal reflex (p<0.001), and finally higher prevalence of LSM $\geq$ 7 kPa + abnormal reflex (p=0.02) (**Figure 2**).

Table 4. Neurological clinical evaluation stratified by FIB-4 and AST/ALT risk scores and liver stiffness measurement.

	Low Risk FIB-4 score (n = 52)	**High Risk FIB-4 score (n = 25)**	**p**	**Low Risk AST/ALT (n = 23)**	**High Risk AST/ALT (n = 60)**	**p**	**LSM < 7 kPa (n = 40)**	**LSM $\geq$ 7 kPa (n = 14)**	**p**
Whit DPN (%)	21 (40.4)	13 (52)	0.336	9 (39.1)	27 (45)	0.629	12 (30)	8 (57.1)	0.073
MNSI-Q score	1.02 ± 1.49	1.68 ± 1.84	0.96	0.7 ± 1.10	1.45 ± 1.77	0.061	1.05 ± 1.39	1.00 ± 1.24	***0.050***
MNSI-Q abnormal (%)	4 (7.7)	4 (16)	0.263	1 (4.3)	8 (13.3)	0.239	3 (7.5)	1 (7.1)	0.484
DNI score	1.81 ± 1.69	2.20 ± 1.77	0.351	1.85 ± 1.88	1.95 ± 1.74	0.815	1.42 ± 1.76	2.21 ± 1.87	0.161
DNI abnormal (%)	18 (34.6)	12 (48)	0.259	8 (34.8)	23 (38.3)	0.765	10 (25)	6 (42.9)	0.083
MDNS	4.81 ± 5.20	6.40 ± 5.87	0.231	5.09 ± 5.30	5.35 ± 5.46	0.843	3.63 ± 4.38	7.93 ± 6.14	***0.006***
MDNS abnormal (%)	14 (26.9)	10 (40)	0.246	7 (30.4)	18 (30)	0.969	7 (17.5)	6 (42.9)	***0.050***
Abnormal vibration sensation at great toe (%)	23 (44.2)	12 (48)	0.756	10 (43.5)	26 (43.3)	0.990	11 (27.5)	8 (57.1)	***0.025***
Abnormal 10-g monofilament test at great toe (%)	6 (11.5)	4 (16)	0.586	2 (8.7)	8 (13.3)	0.561	2 (5)	4 (28.6)	0.063
Abnormal pinprick test (%)	5 (9.6)	2 (8)	0.817	4 (17.4)	4 (6.7)	0.138	2 (5)	2 (14.3)	0.301
Abnormal strength evaluation (%)	6 (11.5)	4 (16)	0.586	3 (13)	8 (13.3)	0.972	2 (5)	2 (14.3)	0.101
Abnormal reflex test at upper	30 (57.7)	17 (68)	0.385	17 (73.9)	33 (55)	0.115	18 (45)	10 (71.4)	***0.042***

and lower limbs (%)									

[Footnotes: AST/ALT, aspartate aminotransferase/alanine aminotransferase ratio; DNI, Diabetic Neuropathy Index; DPN, diabetic peripheral neuropathy; FIB-4, Fibrosis-4; LSM, liver stiffness measurement; MDNS, Michigan Diabetic Neuropathy Score; MNSI-Q, Questionnaire of the Michigan Neuropathy Screening Instrument].

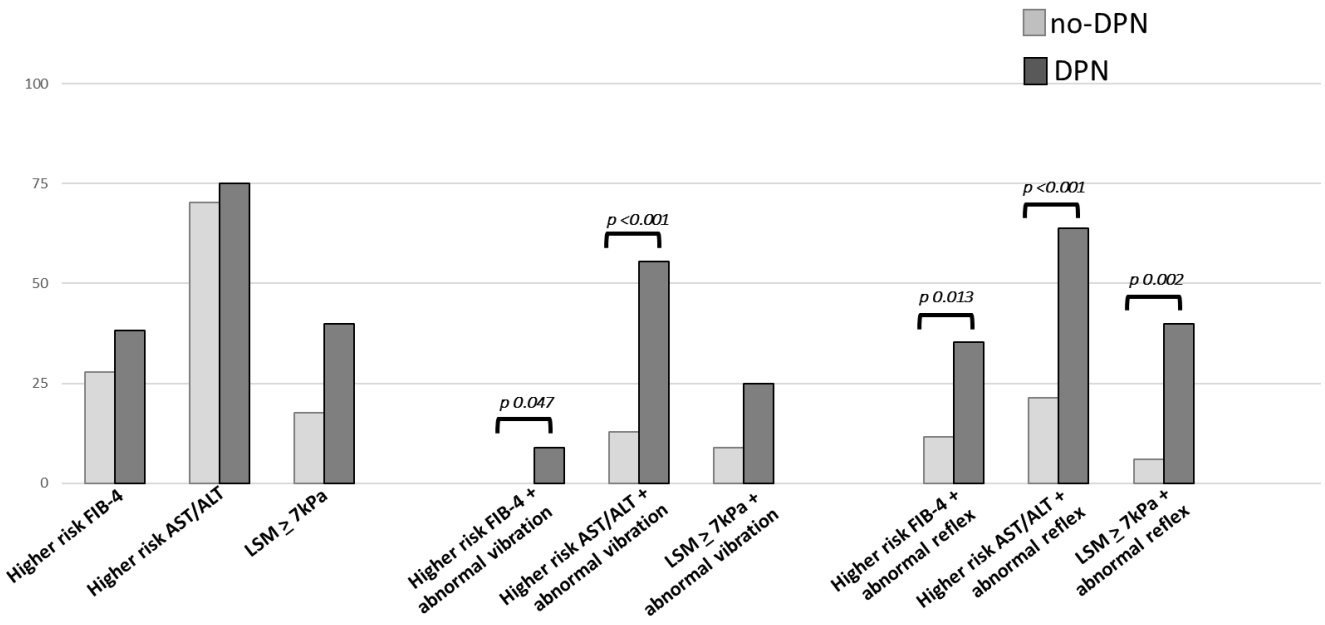
Table 5. Prevalence of combination of high risk of liver fibrosis evaluated by FIB-4 score and AST/ALT ratio and abnormal neurological examination according to the clinical diagnosis of DPN.

	no-DPN	**DPN**	**p**
Higher* risk FIB-4 + abnormal vibration sensation (%)	0	8.83	***0.047***
Higher* risk FIB-4 + abnormal reflex (%)	11.6	35.3	***0.013***
Higher* risk AST/ALT + abnormal vibration sensation (%)	12.8	55.6	***<0.001***
Higher* risk AST/ALT+ abnormal reflex (%)	21.3	63.9	***<0.001***
LSM $\geq$ 7 kPa + abnormal vibration sensation (%)	8.8	25	0.126
LSM $\geq$ 7 kPa + abnormal reflex (%)	5.9	40	***0.02***

****** intermediate and high risk*

[Footnotes: AST/ALT, aspartate aminotransferase/alanine aminotransferase ratio; DPN, diabetic peripheral neuropathy; FIB-4, Fibrosis-4; LSM, liver stiffness measurement; kPa, kilopascals].

Figure 2. Prevalence of combination of high risk of liver fibrosis evaluated by FIB-4 score and AST/ALT ratio and abnormal neurological examination (vibration perception and tendon reflexes) according to the clinical diagnosis of DPN.



[Footnotes: FIB-4, Fibrosis-4; AST/ALT, aspartate aminotransferase/alanine aminotransferase ratio; DPN, diabetic peripheral neuropathy; LSM, liver stiffness measurement; kPa, kilopascals].

4.5. CORRELATION BETWEEN FIBROSIS INDICES AND SYMPTOMS AND SIGNS OF DIABETIC PERIPHERAL NEUROPATHY

Finally, MNSI score was significantly directly related to AST/ALT ratio (Rho: 0.27, p=0.013), while MDNS was significantly directly related to LSM (Rho: 0.281, p=0.026) (**Table 6**). Given the relationship between most NITs and age, BMI, duration of diabetes and HbA1c, a multivariate regression analysis was performed also including these measurements as independent variables. In this analysis, the correlation between score of MNSI-Q and value of AST/ALT ratio remained significant (**Table 6**).

Table 6. Correlation between liver fibrosis indices and symptoms and signs of DPN.

		MNSI-Q score	DNI score	MDNS	FIB-4 score	AST/ALT score	LSM
MNSI-Q score	*Rho*	---	0.243	0.413	0.184	0.270	0.043
	p-value	---	***0.024***	***<0.001***	0.109	**0.013***	0.760
DNI score	*Rho*	0.243	---	0.731	0.158	0.094	0.108
	p-value	***0.024***	---	***<0.001***	0.170	0.399	0.439
MDNS	*Rho*	0.413	0.731	---	0.183	0.120	0.304
	p-value	***<0.001***	***<0.001***	---	0.112	0.281	***0.026***
FIB-4 score	*Rho*	0.184	0.158	0.183	---	0.615	0.197
	p-value	0.109	0.170	0.112	---	***<0.001***	0.185
AST/ALT score	*Rho*	0.270	0.094	0.120	0.615	---	0.277
	p-value	***0.013***	0.399	0.281	***<0.001***	---	***0.049***
LSM	*Rho*	0.043	0.108	0.304	0.197	0.277	---
	p-value	0.760	0.439	***0.026***	0.185	***0.049***	---

** The correlation persisted after correction for age, diabetes duration, HbA1c, and BMI.*

[Footnotes: AST/ALT, aspartate aminotransferase/alanine aminotransferase ratio; DNI, Diabetic Neuropathy Index; FIB-4, Fibrosis-4; LSM, liver stiffness measurement; MDNS, Michigan Diabetic Neuropathy Score; MNSI-Q, Questionnaire of the Michigan Neuropathy Screening Instrument].

4.6 EXPLORATORY RESULTS

Diabetic autonomic neuropathy (DAN) is a form of diabetic neuropathy, it can affect the entire autonomic nervous system, both the parasympathetic and the sympathetic branch, and it leads to dysfunction of the cardiovascular, gastrointestinal, and genitourinary systems, sudomotor dysfunction, and impairment of neurovascular function. Cardiovascular autonomic neuropathy (CAN) is one of the main forms of DAN, and it is defined as the impairment of autonomic control of the cardiovascular system in the setting of diabetes after exclusion of other causes (55). Association among non-invasive biomarkers of liver fibrosis and direct liver stiffness measurement and CAN risk score was investigated as exploratory outcome.

We collected data from 71 T2DM individuals of the total sample who had undergone CAN risk assessment. To define the risk of CAN, test of orthostatic hypotension, study of autonomic symptoms and risk score have been adopted. Orthostatic hypotension was defined as borderline in the case of a sustained reduction in systolic blood pressure of ≥20 mmHg and <30 mmHg, and as confirmed if ≥30 mmHg when moving from a supine to a standing position. Furthermore, autonomic symptoms were assessed using the COMPASS-31 questionnaire (56), a validated questionnaire developed for the assessment of dysautonomic symptoms and widely employed for screening of CAN in diabetes. Finally, the risk of CAN has been studied using the CAN risk score, scoring system primarily based on clinical variables and enables stratification of patients according to their risk of developing CAN (57). The CAN risk score was calculated for each participant and considered positive if >3 or >4, depending on the threshold applied (57, 58).

Table 7 shows the values of non-invasive biomarkers of liver fibrosis and direct liver stiffness measurement in the overall sample of 71 subjects who underwent CAN risk evaluation. When stratified by CAN risk, no significant variation was detected in fibrosis indexes (FIB-4 score, NFS, APRI, AST/ALT ratio, and LSM) (Table 8).

Table 7. Non-invasive biomarkers of liver fibrosis and liver stiffness measurement in seventy-one subjects underwent CAN risk evaluation.

Non-invasive biomarkers of fibrosis		
	FIB-4 score (mean ± SD)	1,44±0,85
	FIB-4 higher risk***** score (%)	5/61 (8,2)
	NFS (mean ± SD)	-0,04±1,16

	NFS higher risk* score (%)	11/52 (21,1)
	AST/ALT ratio (mean ± SD)	1,01±0,52
	AST/ALT ratio higher risk* score (%)	9/67 (13,4)
	APRI score (mean ± SD)	0,35±0,21
	APRI higher risk* score (%)	6/62 (9,7)
Instrumental evaluation of fibrosis		
	Mean LSM (kPa) (n = 54)	6.52 ± 3.45
	With LSM > 7 kPa (%) (n = 54)	15/59 (25,4)

** Intermediate and high risk*

[Footnotes: APRI, aspartate aminotransferase to platelet ratio index; AST/ALT, aspartate aminotransferase/alanine aminotransferase ratio; DPN, diabetic peripheral neuropathy; FIB-4, Fibrosis-4; kPa, kilopascal; LSM, liver stiffness measurement; NFS, NAFLD (non-alcoholic fatty liver disease) fibrosis score].

Table 8. NITs and LSM measurement according to CAN risk score > 4 in seventy-one subjects underwent CAN risk evaluation.

		CAN risk score < 4 (n = 47)	**CAN risk score ≥ 4** (n = 24)	**p**
Non-invasive biomarkers of fibrosis				
	FIB-4 score (mean ± SD)	1,35±0,70	1,66±1,03	0,22
	FIB-4 higher risk* score (%)	7,89	9,52	0,83
	NFS (mean ± SD)	-0,10±1,18	0,10±1,15	0,56
	NFS higher risk* score (%)	18,18	27,78	0,43
	AST/ALT ratio (mean ± SD)	0,98±0,37	1,06±0,73	0,63
	AST/ALT ratio higher risk* score (%)	10	20,83	0,23
	APRI score (mean ± SD)	0,33±0,21	0,38±0,21	0,39
	APRI higher risk* score (%)	5,13	14,29	0,22
Instrumental evaluation of fibrosis				
	Mean LSM (kPa) (n = 54)	6,58±3,74	6,86±2,96	0,77
	With LSM > 7 kPa (%) (n = 54)	23,07	29,41	0,61

** Intermediate and high risk*

[Footnotes: APRI, aspartate aminotransferase to platelet ratio index; AST/ALT, aspartate aminotransferase/alanine aminotransferase ratio; DPN, diabetic peripheral neuropathy; FIB-4, Fibrosis-4; kPa, kilopascal; LSM, liver stiffness measurement; NFS, NAFLD (non-alcoholic fatty liver disease) fibrosis score]

5. DISCUSSION

In this study we investigated the association between liver fibrosis (NITs, indices of fibrosis risk and measurement of stiffness) and DPN. Liver fibrosis detected by LSM seems more prevalent in DPN^+ subjects, even if this result is not statistically significance, maybe because of the small sample size (40 *vs* 17.5%, p=0.073). Moreover, the fibrosis indices (FIB-4 score and ASL/ALT ratio) were higher in DPN^+ population, suggesting an association between risk of fibrosis and DPN. Indeed, higher MNSI-Q score (p=0.05) and MDNS score (p=0.006), abnormal result in MDNS (p=0.05), abnormal vibration sensation at great toe (p=0.025), and abnormal reflex test at upper and lower limbs (p=0.042) were associated to a LSM $\geq$ 7 kPa, suggestive of liver fibrosis. Giving these results, it seems that in subjects with T2DM and liver fibrosis to detect DPN is more useful to investigate the signs of DPN, especially the vibratory sensation at great toe and the reflexes at upper and lower limbs, indicative of large nerve fibers involvement. Then, we evaluated the different signs of DPN in combination with the fibrosis indices FIB-4 score and AST/ALT ratio, and LSM $\geq$7 kPa. We demonstrated that DPN^+ individuals show higher risk FIB-4 score + abnormal vibration sensation (p=0.047), higher risk FIB-4 + abnormal reflex (p=0.013), higher risk AST/ALT ratio + abnormal vibration sensation (p<0.001), higher risk AST/ALT ratio + abnormal reflex (p<0.001), and finally higher prevalence of LSM $\geq$ 7 kPa + abnormal reflex (p=0.02). The meaning of these results is important because the simple qualitative evaluation of vibration perception or tendon reflexes would be useful and effective in identifying DPN in T2DM with MASLD at high risk of fibrotic evolution. Finally, MNSI, the tool used to evaluate DPN symptoms is significantly related to AST/ALT ratio (Rho: 0.27, p=0.013), and MDNS, the tools used to detect DPN signs, is directly

related to LSM (Rho: 0.281, p=0.026), suggesting that DPN symptoms and signs correlates with fibrosis indices and LSM.

The association between liver fibrosis and DPN was already described in literature: a multicenter study on 442 subjects with T2DM demonstrated that significant liver fibrosis is associated with an increased likelihood of having myocardial infarction (adjusted-OR 6.61, 95%CI 1.66-37.4), peripheral polyneuropathy (adjusted-OR 4.55, 95%CI 1.25-16.6), CKD (adjusted-OR 4.54, 95%CI 1.24-16.6), retinopathy (adjusted-OR 1.81, 95%CI 1.62-1.97), independently of cardiometabolic risk factors, diabetes-related variables, and other potential confounders (33). Moreover, Williams *et al* in 2015 reported an association between elevated lower-limb vibration perception threshold (VPT) and non-invasive measures of liver fibrosis (59). In detail, higher VPT was associated with high risk of MASLD fibrosis score in T1- and T2- DM and with liver fibrosis evaluated by LSM in T2DM (59). This result is in line with our findings. Finally, Huang *et al* confirmed the correlation between DPN and liver fibrosis in a cohort of 520 individuals with T2DM undergoing a nerve conduction study for neuropathy and FibroTouch for liver steatosis and fibrosis diagnosis (60).

Considering a retrospective and observational design of this study, it can't produce generalisable or transferable findings. Indeed, our clinical observations are not able to prove the causality of association between PDN and liver fibrosis. However, the findings may tell us about trends and associations and be used to generate a hypothesis. Further limitations of this study are the monocentric nature and the small size of the sample. Considering the prevalence of liver fibrosis and PND in people with T2DM based on

previous literature, the sample size was calculated from n=62 to n=100 using a sample size calculator set to a two-sided alpha cut-off of 5% (0.05) and a power of 80% and a two-sided alpha cut-off of 5% (0.05) and a power of 95%, respectively. However, the non-significant result for difference of LSM ≥7 kPa between DPN+ and DPN-(40% vs 17.5%, p=0.073) in this cohort of 86 subjects may reflect in part the need to increase the number of observations, in particular of the group undergo to Fibroscan that included only 54 measurement, so far. Further consideration should be made regarding the LSM cut-off for the definition of liver fibrosis. According to recent guidelines (61,62), individuals with a LSM ≥8.0 kPa should be considered for referral to liver specialty clinics (gastroenterology or hepatology) for additional assessment, including imaging-based methods such as magnetic resonance elastography (MRE) or multi parametric MRI-derived iron-corrected T1 (cT1). On the other hand, liver biopsy is generally considered when non-invasive assessments are in conclusive or when alternative diagnoses are suspected. However, in our clinical practice, we commonly adopt a lower cut-off of LSM ($\geq$ 7 kPa) to early identify patients who could benefit from a management by a specific interprofessional team, more intensive treatment of risk factors and more active monitoring of condition of fibrosis and comorbidities. Given this clinical bias, we used the lowest LSM value as the screening cut off in our analyses. Using an LSM value $\geq$ 8 kPa led to a further reduction in the sample size, making the trend in the results even more inconsistent.

The strength of our study is the well characterized T2DM population. Moreover, among the strengths of the study, we report the adequate exclusion of possible liver disease from excessive alcohol consumption. Indeed, in the anamnesis collection, alcohol

consumption habits were appropriately investigated in order to exclude subjects with a likely alcohol-related liver disease (ALD). Specifically, a consumption less to 20 g of alcohol per day in females or less to 30 g of alcohol in males were typically considered acceptable (62). Another strength point of the study is represented by the accurate investigation according to the standard procedures for the clinical diagnosis of PND. Regarding the definition of clinical diagnosis of DPN, it required the presence of at least one abnormality among symptoms in terms of MNSI-Q cut off $\geq$ 4 and/or signs in terms of DNI cut off > 2 or MDNS cut off > 7. The alteration in vibration perception alone or reflexes alone, although they are important part of clinical investigation, are not sufficient to DPN diagnosis in absence of total positivity of DNI and/or MDNS. On the other hand, the results of the analysis of our observations highlight that in subjects at high risk of liver fibrosis, even the single detection of alterations in vibratory sensitivity and/or osteotendinous reflexes is sufficient and accurate to identify the neurological complication.

Despite these limitations, this study suggests the need for special attention to DPN in individuals with T2DM and liver disease. This finding requires validation in larger cohort; future studies to investigate the molecular mechanism of the association between liver fibrosis and DPN are necessary. Finally, prospective and multicenter studies could strengthen the data reported in the current research.

6. CONCLUSION

We documented the association between higher fibrosis indices and presence of DPN, in particular higher FIB-4 score and AST/ALT ratio were found to be associated to the presence of DPN. In particular, the presence of liver fibrosis was found to be associated to the signs of DPN, especially the abnormal vibration sensation at great toe and the abnormal reflex test at upper and lower limbs, indicative of large nerve fibers involvement. Moreover, the presence of an indeterminate or high risk on FIB-4 score or ASL/ALT ratio or a LSM $\geq$7 kPa in combination with one sign of DPN between abnormal vibration sensation at great toe and the abnormal reflex test at upper and lower limbs was found to be associated with the presence of DPN. In clinical practice, for subjects with T2DM and liver fibrosis or at risk of fibrosis (calculated with FIB-4 score or AST/ALT ratio), it can be useful to perform vibration sensation at great toe and abnormal reflex to identify subjects with DPN. On the other hand, in subjects with T2DM and DPN could be of use to calculate fibrosis indices, in particular FIB-4 score and AST/ALT ratio to detect subjects with fibrosis.

In conclusion, this cross-sectional study systematically investigates, for the first time, the screening value of combining non-invasive liver fibrosis indicators (FIB-4 and AST/ALT ratio) with vibration perception or reflex tests for DPN in T2DM patients with MASLD. These findings allow to propose a combined screening model of "high-risk liver fibrosis + abnormal simple neurological signs" with potential clinical utility.

7. BIBLIOGRAPHY

1. American Diabetes Association Professional Practice C. 12. Retinopathy, Neuropathy, and Foot Care: Standards of Care in Diabetes-2024. Diabetes Care. 2024;47(Suppl 1):S231-S43.
2. Pop-Busui R, Boulton AJ, Feldman EL, Bril V, Freeman R, Malik RA, et al. Diabetic Neuropathy: A Position Statement by the American Diabetes Association. Diabetes Care. 2017;40(1):136-54.
3. Feldman EL, Stevens MJ, Thomas PK, Brown MB, Canal N, Greene DA. A practical two-step quantitative clinical and electrophysiological assessment for the diagnosis and staging of diabetic neuropathy. Diabetes Care. 1994;17(11):1281-9.
4. Fedele D, Comi G, Coscelli C, Cucinotta D, Feldman EL, Ghirlanda G, et al. A multicenter study on the prevalence of diabetic neuropathy in Italy. Italian Diabetic Neuropathy Committee. Diabetes Care. 1997;20(5):836-43.
5. Dyck PJ, Albers JW, Andersen H, Arezzo JC, Biessels GJ, Bril V, et al. Diabetic polyneuropathies: update on research definition, diagnostic criteria and estimation of severity. Diabetes Metab Res Rev. 2011;27(7):620-8.
6. Rinella ME, Lazarus JV, Ratziu V, Francque SM, Sanyal AJ, Kanwal F, et al. A multisociety Delphi consensus statement on new fatty liver disease nomenclature. J Hepatol. 2023;79(6):1542-56.
7. Forlano R, Stanic T, Jayawardana S, Mullish BH, Yee M, Mossialos E, et al. A prospective study on the prevalence of MASLD in people with type-2 diabetes in the community. Cost effectiveness of screening strategies. Liver Int. 2024;44(1):61-71.
8. Hagstrom H, Vessby J, Ekstedt M, Shang Y. 99% of patients with NAFLD meet MASLD criteria and natural history is therefore identical. J Hepatol. 2024;80(2):e76-e7.
9. Chan WK, Chuah KH, Rajaram RB, Lim LL, Ratnasingam J, Vethakkan SR. Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD): A State-of-the-Art Review. J Obes Metab Syndr. 2023;32(3):197-213.
10. Le MH, Yeo YH, Li X, Li J, Zou B, Wu Y, et al. 2019 Global NAFLD Prevalence: A Systematic Review and Meta-analysis. Clin Gastroenterol Hepatol. 2022;20(12):2809-17 e28.
11. Wong VW, Ekstedt M, Wong GL, Hagstrom H. Changing epidemiology, global trends and implications for outcomes of NAFLD. J Hepatol. 2023;79(3):842-52.
12. Cusi K, Isaacs S, Barb D, Basu R, Caprio S, Garvey WT, et al. American Association of Clinical Endocrinology Clinical Practice Guideline for the Diagnosis and Management of Nonalcoholic Fatty Liver Disease in Primary Care and Endocrinology Clinical Settings: Co-Sponsored by the American Association for the Study of Liver Diseases (AASLD). Endocr Pract. 2022;28(5):528-62.
13. Singh S, Allen AM, Wang Z, Prokop LJ, Murad MH, Loomba R. Fibrosis progression in nonalcoholic fatty liver vs nonalcoholic steatohepatitis: a systematic review and meta-analysis of paired-biopsy studies. Clin Gastroenterol Hepatol. 2015;13(4):643-54 e1-9; quiz e39-40.
14. Chan WL, Chong SE, Chang F, Lai LL, Chuah KH, Nik Mustapha NR, et al. Long-term clinical outcomes of adults with metabolic dysfunction-associated fatty liver disease: a single-centre prospective cohort study with baseline liver biopsy. Hepatol Int. 2023;17(4):870-81.

15. Rinella ME, Neuschwander-Tetri BA, Siddiqui MS, Abdelmalek MF, Caldwell S, Barb D, et al. AASLD Practice Guidance on the clinical assessment and management of nonalcoholic fatty liver disease. Hepatology. 2023;77(5):1797-835.
16. Kahn CR, Wang G, Lee KY. Altered adipose tissue and adipocyte function in the pathogenesis of metabolic syndrome. J Clin Invest. 2019;129(10):3990-4000.
17. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. J Clin Invest. 2005;115(5):1343-51.
18. Sanders FWB, Acharjee A, Walker C, Marney L, Roberts LD, Imamura F, et al. Hepatic steatosis risk is partly driven by increased de novo lipogenesis following carbohydrate consumption. Genome Biol. 2018;19(1):79.
19. Bril F, Cusi K. Management of Nonalcoholic Fatty Liver Disease in Patients With Type 2 Diabetes: A Call to Action. Diabetes Care. 2017;40(3):419-30.
20. Targher G, Byrne CD, Tilg H. MASLD: a systemic metabolic disorder with cardiovascular and malignant complications. Gut. 2024;73(4):691-702.
21. Younossi ZM, Golabi P, de Avila L, Paik JM, Srishord M, Fukui N, et al. The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: A systematic review and meta-analysis. J Hepatol. 2019;71(4):793-801.
22. Mantovani A, Petracca G, Beatrice G, Tilg H, Byrne CD, Targher G. Non-alcoholic fatty liver disease and risk of incident diabetes mellitus: an updated meta-analysis of 501 022 adult individuals. Gut. 2021;70(5):962-9.
23. Wongtrakul W, Charatcharoenwitthaya N, Charatcharoenwitthaya P. Metabolic dysfunction-associated steatotic liver disease and the risk of mortality in individuals with type 2 diabetes: a systematic review and meta-analysis. Eur J Gastroenterol Hepatol. 2024;36(4):351-8.
24. Targher G, Lonardo A, Byrne CD. Nonalcoholic fatty liver disease and chronic vascular complications of diabetes mellitus. Nat Rev Endocrinol. 2018;14(2):99-114.
25. Mantovani A, Csermely A, Petracca G, Beatrice G, Corey KE, Simon TG, et al. Non-alcoholic fatty liver disease and risk of fatal and non-fatal cardiovascular events: an updated systematic review and meta-analysis. Lancet Gastroenterol Hepatol. 2021;6(11):903-13.
26. Mantovani A, Zaza G, Byrne CD, Lonardo A, Zoppini G, Bonora E, et al. Nonalcoholic fatty liver disease increases risk of incident chronic kidney disease: A systematic review and meta-analysis. Metabolism. 2018;79:64-76.
27. Deravi N, Dehghani Firouzabadi F, Moosaie F, Asadigandomani H, Arab Bafrani M, Yoosefi N, et al. Non-alcoholic fatty liver disease and incidence of microvascular complications of diabetes in patients with type 2 diabetes: a prospective cohort study. Front Endocrinol (Lausanne). 2023;14:1147458.
28. Zou Y, Zhao L, Zhang J, Wang Y, Wu Y, Ren H, et al. Metabolic-associated fatty liver disease increases the risk of end-stage renal disease in patients with biopsy-confirmed diabetic nephropathy: a propensity-matched cohort study. Acta Diabetol. 2023;60(2):225-33.
29. Wen X, Zhou X, Chen D, Cheng J, Ji L. Association Between Non-Alcoholic Fatty Liver Disease and Diabetes-Related Microvascular Complications: A Retrospective Cross-Sectional Study of Hospitalized Patients. Endocr Pract. 2022;28(3):304-9.

30. Targher G, Bertolini L, Rodella S, Zoppini G, Lippi G, Day C, et al. Non-alcoholic fatty liver disease is independently associated with an increased prevalence of chronic kidney disease and proliferative/laser-treated retinopathy in type 2 diabetic patients. Diabetologia. 2008;51(3):444-50.
31. Greco C, Nascimbeni F, Carubbi F, Andreone P, Simoni M, Santi D. Association of Nonalcoholic Fatty Liver Disease (NAFLD) with Peripheral Diabetic Polyneuropathy: A Systematic Review and Meta-Analysis. J Clin Med. 2021;10(19).
32. Ebert T, Widman L, Stenvinkel P, Hagstrom H. Increased risk for microvascular outcomes in NAFLD-A nationwide, population-based cohort study. J Intern Med. 2023;294(2):216-27.
33. Mikolasevic I, Rahelic D, Turk-Wensween T, Ruzic A, Domislovic V, Hauser G, et al. Significant liver fibrosis, as assessed by fibroscan, is independently associated with chronic vascular complications of type 2 diabetes: A multicenter study. Diabetes Res Clin Pract. 2021;177:108884.
34. Lombardi R, Airaghi L, Targher G, Serviddio G, Maffi G, Mantovani A, et al. Liver fibrosis by FibroScan((R)) independently of established cardiovascular risk parameters associates with macrovascular and microvascular complications in patients with type 2 diabetes. Liver Int. 2020;40(2):347-54.
35. Arab JP, Karpen SJ, Dawson PA, Arrese M, Trauner M. Bile acids and nonalcoholic fatty liver disease: Molecular insights and therapeutic perspectives. Hepatology. 2017;65(1):350-62.
36. Guo C, Chen WD, Wang YD. TGR5, Not Only a Metabolic Regulator. Front Physiol. 2016;7:646.
37. Deutschmann K, Reich M, Klindt C, Dröge C, Spomer L, Häussinger D, et al. Bile acid receptors in the biliary tree: TGR5 in physiology and disease. Biochim Biophys Acta Mol Basis Dis. 2018;1864(4 Pt B):1319-25.
38. Hu X, Yan J, Huang L, Araujo C, Peng J, Gao L, et al. INT-777 attenuates NLRP3-ASC inflammasome-mediated neuroinflammation via TGR5/cAMP/PKA signaling pathway after subarachnoid hemorrhage in rats. Brain Behav Immun. 2021;91:587-600.
39. Lewis ND, Patnaude LA, Pelletier J, Souza DJ, Lukas SM, King FJ, et al. A GPBAR1 (TGR5) small molecule agonist shows specific inhibitory effects on myeloid cell activation in vitro and reduces experimental autoimmune encephalitis (EAE) in vivo. PLoS One. 2014;9(6):e100883.
40. Zhou Y, Notterpek L. Promoting peripheral myelin repair. Exp Neurol. 2016;283(Pt B):573-80.
41. Liu X, Guan J, Wu Z, Xu L, Sun C. The TGR5 Agonist INT-777 Promotes Peripheral Nerve Regeneration by Activating cAMP-dependent Protein Kinase A in Schwann Cells. Mol Neurobiol. 2023;60(4):1901-13.
42. easloffice@easloffice.eu EAftSotLEa, Panel CPG, Chair:, representative: EGB, members: P. EASL Clinical Practice Guidelines on non-invasive tests for evaluation of liver disease severity and prognosis - 2021 update. J Hepatol. 2021;75(3):659-89.
43. (EASL) EAftSotL, (EASD) EAftSoD, (EASO) EAftSoO. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. J Hepatol. 2016;64(6):1388-402.
44. Salomone F, Micek A, Godos J. Simple Scores of Fibrosis and Mortality in Patients with NAFLD: A Systematic Review with Meta-Analysis. J Clin Med. 2018;7(8).

45. De Vincentis A, Costanzo L, Vespasiani-Gentilucci U, Picardi A, Bandinelli S, Ferrucci L, et al. Association between non-invasive liver fibrosis scores and occurrence of health adverse outcomes in older people. Dig Liver Dis. 2019;51(9):1330-6.
46. Önnerhag K, Hartman H, Nilsson PM, Lindgren S. Non-invasive fibrosis scoring systems can predict future metabolic complications and overall mortality in non-alcoholic fatty liver disease (NAFLD). Scand J Gastroenterol. 2019;54(3):328-34.
47. Hagström H, Nasr P, Ekstedt M, Stål P, Hultcrantz R, Kechagias S. Accuracy of Noninvasive Scoring Systems in Assessing Risk of Death and Liver-Related Endpoints in Patients With Nonalcoholic Fatty Liver Disease. Clin Gastroenterol Hepatol. 2019;17(6):1148-56.e4.
48. Kim K, Oh TJ, Cho HC, Lee YK, Ahn CH, Koo BK, et al. Liver fibrosis indices are related to diabetic peripheral neuropathy in individuals with type 2 diabetes. Sci Rep. 2021;11(1):24372.
49. Leite NC, Cardoso CRL, Salles GF. Importance of non-invasive liver fibrosis scores for mortality and complications development in individuals with type 2 diabetes. J Diabetes Complications. 2021;35(5):107879.
50. ElSayed NA, Aleppo G, Aroda VR, Bannuru RR, Brown FM, Bruemmer D, et al. 2. Classification and Diagnosis of Diabetes: Standards of Care in Diabetes-2023. Diabetes Care. 2023;46(Suppl 1):S19-S40.
51. Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, et al. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. Hepatology. 2006;43(6):1317-25.
52. Angulo P, Hui JM, Marchesini G, Bugianesi E, George J, Farrell GC, et al. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. Hepatology. 2007;45(4):846-54.
53. Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology. 2003;38(2):518-26.
54. Wong VW, Vergniol J, Wong GL, Foucher J, Chan HL, Le Bail B, et al. Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. Hepatology. 2010;51(2):454-62.
55. Spallone V, Ziegler D, Freeman R, Bernardi L, Frontoni S, Pop-Busui R, et al. Cardiovascular autonomic neuropathy in diabetes: clinical impact, assessment, diagnosis, and management. Diabetes Metab Res Rev. 2011;27(7):639-53.
56. Greco C, Di Gennaro F, D'Amato C, Morganti R, Corradini D, Sun A, et al. Validation of the Composite Autonomic Symptom Score 31 (COMPASS 31) for the assessment of symptoms of autonomic neuropathy in people with diabetes. Diabet Med. 2017;34(6):834-8.
57. Menduni M, D'Amato C, Leoni M, Izzo V, Staltari M, Greco C, et al. Clinical scoring systems for the risk of cardiovascular autonomic neuropathy in type 1 and type 2 diabetes: A simple tool. J Peripher Nerv Syst. 2022;27(4):259-70.
58. Pertile P, D'Ippolito I, De Santis B, Andreadi A, Lauro D, Spallone V. External validation of a clinical risk score for the presence of cardiovascular autonomic neuropathy in type 1 diabetes. J Diabetes Complications. 2025;39(7):109066.
59. Williams KH, Burns K, Constantino M, Shackel NA, Prakoso E, Wong J, et al. An association of large-fibre peripheral nerve dysfunction with non-invasive measures of liver

fibrosis secondary to non-alcoholic fatty liver disease in diabetes. J Diabetes Complications. 2015;29(8):1240-7.
60. Huang J, Li R, Liu N, Yi N, Zheng H, Zhang Q, et al. Liver fibrosis is independently associated with diabetic peripheral neuropathy in type 2 diabetes mellitus. J Diabetes Investig. 2021;12(11):2019-27.

61. Cusi K, Abdelmalek MF, Apovian CM, Balapattabi K, Bannuru RR, Barb D, Bardsley JK, Beverly EA, Corbin KD, ElSayed NA, Isaacs S, Kanwal F, Pekas EJ, Richardson CR, Roden M, Sanyal AJ, Shubrook JH, Younossi ZM, Bajaj M. Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) in People With Diabetes: The Need for Screening and Early Intervention. A Consensus Report of the American Diabetes Association. Diabetes Care. 2025 Jul 1;48(7):1057-1082. doi: 10.2337/dci24-0094. PMID: 40434108.

62. European Association for the Study of the Liver (EASL); European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines on the management of metabolic dysfunction-associated steatotic liver disease (MASLD). J Hepatol. 2024 Sep;81(3):492-542. doi: 10.1016/j.jhep.2024.04.031. Epub 2024 Jun 7. PMID: 38851997.