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Severity of Nonalcoholic Fatty Liver Disease in Type 2 Diabetes Mellitus: Relationship between Nongenetic Factors and PNPLA3/HSD17B13 Polymorphisms

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Background: The prevalence of nonalcoholic fatty liver disease (NAFLD) in patients with type 2 diabetes mellitus (T2DM) is high, though its severity is often underestimated. Our aim is to provide an estimate of the prevalence of severe NAFLD in T2DM and identify its major predictors.
Methods: T2DM patients (n=328) not previously known to have NAFLD underwent clinical assessment, transient elastography with measure of liver stiffness (LS) and controlled attenuation parameter (CAP), and genotyping for patatin like phospholipase domain containing 3 (*PNPLA3*) and 17β-hydroxysteroid-dehydrogenase type 13 (*HSD17B13*).
Results: Median LS was 6.1 kPa (4.9 to 8.6). More than one-fourth patients had advanced liver disease, defined as LS ≥7.9 kPa (n=94/238, 29%), and had a higher body mass index (BMI) than those with a LS <7.9 kPa. Carriage of the G allele in the *PNPLA3* gene was associated with higher LS, being 5.9 kPa (4.7 to 7.7) in C/C homozygotes, 6.1 kPa (5.2 to 8.7) in C/G heterozygotes, and 6.8 kPa (5.8 to 9.2) in G/G homozygotes (P=0.01). This trend was absent in patients with ≥1 mutated *HSD17B13* allele. In a multiple linear regression model, BMI and *PNPLA3* genotype predicted LS, while age, gender, disease duration, and glycosylated hemoglobin did not fit into the model. None of these variables was confirmed to be predictive among carriers of at least one *HSD17B13* mutated allele. There was no association between CAP and polymorphisms of *PNPLA3* or *HSD17B13*.
Conclusion: Advanced NAFLD is common among T2DM patients. LS is predicted by both BMI and *PNPLA3* polymorphism, the effect of the latter being modulated by mutated *HSD17B13*.

Keywords: Adiponutrin; Body mass index; Diabetes mellitus, type 2; Fibrosis; Non-alcoholic fatty liver disease

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the name given to a spectrum of liver disorders, histologically categorized as simple (i.e., isolated) steatosis or nonalcoholic steatohepatitis (NASH) with or without fibrosis, often resulting in cirrhosis and hepatocellular carcinoma (HCC) [1]. Like its major risk factors, obesity and type 2 diabetes mellitus (T2DM), NAFLD results from the interaction between environmental and genetic factors [2]. Specifically, variants in two genes coding for

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proteins involved in lipolysis and lipogenesis, namely adiponutrin (also known as patatin like phospholipase domain containing 3 [*PNPLA3*]) and, more recently, 17β-hydroxysteroid-dehydrogenase type 13 (*HSD17B13*), have been shown to play a significant role in NAFLD pathogenesis and progression [3].

There is an ongoing debate regarding whether screening campaigns should be conducted to identify NAFLD individuals [4]. The overwhelmingly high prevalence, the relatively low risk of progression to significant liver disease for most NAFLD patients, and the paucity of treatment options other than lifestyle measures—notoriously difficult to adhere to—make large-scale screenings for NAFLD highly unsuitable for the general population. Thus, screening efforts focused on individuals at higher risk of liver disease progression are likely to be far more rewarding. In this regard, T2DM patients are known not only to be at high risk for NAFLD development [5] but also to display a progression rate toward significant liver fibrosis double that observed in other NAFLD patients [6]. However, it is still unclear what are the determinants of this increased risk given the multifactorial etiology of diabetes [7]. Certainly, the perception of NAFLD among physicians caring for patients with T2DM varies widely, with normal aminotransferase test being a predictor for non-referral or no further diagnostic exploration of T2DM patients among endocrinologists [8].

In recent years, the way clinicians approach a patient with suspected liver disease has been changed by the advent of transient elastography (TE), a non-invasive technique which—by extrapolating the likelihood of fibrosis from liver stiffness (LS)—has greatly reduced the indications for a liver biopsy, perceived by many as unduly aggressive and costly [9]. Conveniently, new TE machines have been implemented with a software able to quantify liver fat accurately, by measuring the controlled attenuation parameter (CAP). CAP identifies steatosis independently of the presence of fibrosis, which is not the case for ultrasound (US) [10]. Systematically performing TE with CAP measurement on patients with T2DM may thus offer the opportunity of unveiling the true burden of liver disease in this condition, thereby increasing the awareness of its existence among non-hepatologists. At the same time, it makes possible exploring genetic and environmental risk factors associated with NAFLD progression towards liver fibrosis among diabetics. With these aims in mind we performed the present study.

METHODS

Patients

All consecutive adult patients with T2DM evaluated at the diabetes clinic of the Madonna del Popolo Hospital in Omegna and the Castelli Hospital in Verbania, in Northern Italy, from 1st March 2017 to 1st March 2018, were offered to participate to this study, according to the protocol approved by the local Ethical Committee (IRB No. 176/18). A written informed consent was obtained by all participants ($n=328$, of whom $n=243$ were males). The study was approved by the local Ethical Committee (Comitato Etico Interaziendale A.O.U. "Maggiore della Carità," ASL BI, ASL NO, ASL VCO) and conducted in strict accordance with the principles of the Declaration of Helsinki.

The inclusion criteria were as follows: age ≥18 years and T2DM in pharmacological treatment. The exclusion criteria were as follows: positive serology for hepatitis C virus (HCV) infection, chronic hepatitis related to hepatitis B virus infection, concomitance of other causes of chronic liver disease (e.g., autoimmune hepatitis, hemochromatosis, cholestatic liver disease, and drug damage), presence of focal liver lesions of suspected malignant origin, excess consumption of alcoholic beverages (≥3 drinks/day). Stage 2 or higher obesity (body mass index [BMI] ≥35 kg/m^2), and inability to obtain valid TE measurements. We screened 432 patients. After applying the above mentioned criteria, 328 patients were enrolled. At enrolment, a blood sample was drawn, and the following data were registered:

(1) Age and gender

(2) Anthropometric data: these were collected with patients wearing only light underwear: the BMI was calculated as body weight divided by the square of the height (kg/m^2) and interpreted according to World Health Organization classification.

(3) A biochemistry panel, including total cholesterol, high density lipoprotein and low density lipoprotein cholesterol, and triglycerides, all measured with enzymatic methods (ADVIA; Siemens, Leverkusen, Germany). Furthermore, glycosylated hemoglobin (HbA1c) was determined by high-pressure chromatography (Variant Biorad II; Biorad, Hercules, CA, USA). Plasma glucose concentration was measured by hexokinase (ADVIA). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase were measured with enzymatic methods

(Advia 1800 Chemistry System, Siemens).

NAFLD evaluation

All patients underwent US examination of the liver performed by a single expert clinician. A sagittal sonographic plane of section demonstrating liver parenchyma and the right kidney was selected to determine liver echogenicity and evaluated as follows [11]:

(1) No steatosis: liver echogenicity comparable to the right kidney.
(2) Grade 1: slightly increased liver echogenicity with respect to the right kidney; echogenicity of the intrahepatic vessel walls and diaphragm was well visualized.
(3) Grade 2: moderate increase of liver echogenicity with respect to the right kidney, with slight decreased visibility of the intrahepatic vessel walls and decreased reflectivity of the diaphragm.
(4) Grade 3: severe steatosis was defined as diffusely increased echogenicity of the liver compared to the right kidney, a lack of visualization of intrahepatic vessel walls, and markedly decreased reflectivity of the hemidiaphragm.

LS was assessed by TE (FibroScan; Echosens, Paris, France). TE was performed as reported by Sandrin et al. [12], using at least 10 valid measurements; examinations were considered reliable when interquartile range was <30% and the success rate was >60%. To define the presence of significant fibrosis, we used the cut-off value of 7.9 kPa, as proposed by others [13]. Moreover, we measured CAP, which allows noninvasive semiquantitative assessment of liver fat content by measuring the attenuation at the center frequency of the FibroScan [14], ensuring that the liver ultrasonic attenuation was obtained simultaneously from the same volume of liver parenchyma as that of LS. CAP values range from 100 to 400 dB/m: the cut-off values we chose to indicate steatosis as absent, mild, moderate and severe were <236, ≥236, ≥270, and ≥302, respectively [15].

NAFLD fibrosis score and fibrosis-4 (FIB-4) were calculated for each patient, as previously reported [16,17]. FIB-4 is an algorithm based on age, AST/ALT plasma levels, and platelets count; NAFLD fibrosis score relies on the same variables plus BMI and albumin concentration, also considering the presence of impaired fasting glucose or diabetes. Both scores were validated for the estimation of fibrotic burden in NAFLD patients.

Genetic studies

Genomic DNA was extracted from whole blood or buffy coat using a commercial kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. DNA was then amplified by polymerase chain reaction (PCR). The PCR primers sequences used for *PNPLA3* amplification were: Forward: 5′-CCTGCAGGCAGGAGATGTGT-3′; reverse: 5′-GCCCTGCTCACTTGGAGAAA-3′. The PCR primers sequences used for *HSD17B13* amplification were: forward: 5′-GTCTGAGGCATGAGAATTGCT-3′; reverse: 5′-GGCCTGTATTGGAGACAGATG -3′. To define the genotype of the two target genes, we performed a restriction fragment length PCR. NLA-III and TRU1I restriction enzymes (ThermoFisher Scientific, Waltham, MA, USA) were used to digest *PNPLA3* and *HSD17B13*, respectively. All samples were amplified twice; when discordant, they were run a third time.

Statistical analysis

Statistical analysis of data was carried out with the statistical software package Stata version 13.1 (StataCorp LP, College Station, TX, USA). The measures of centrality and dispersion of data chosen were medians and interquartile ranges. Continuous variables were compared between groups by the Mann-Whitney test. The nonparametric test chosen to identify a trend across ordered groups was that developed by Cuzick. The exact Fischer's test and the Pearson's chi-square test were used, as appropriate, to explore the associations of categorical variables. Inter-rater agreement was measured by means of κ statistics [18]. The association of hypothetical predictors with LS was modeled through a multiple linear regression model. The chi-square G test "Goodness of Fit" was employed to verify whether the proportions of the two polymorphisms were distributed in patients in accordance with the Hardy-Weinberg equation. The level of significance chosen for all statistical tests was 0.05 (two-tailed).

RESULTS

Characteristics of the study population

The main demographic and clinical characteristics of the study population are shown in Table 1. None of the patients was on high-dose vitamin E, pioglitazone, or sodium/glucose cotransporter 2 (SGLT2) inhibitors when recruited.

Prevalence and main features of NAFLD in T2DM

The median LS was 6.1 kPa, interquartile range 4.9 to 8.6. Fifty-four patients (16%) had either ASTs (n=24, 7%) or ALTs

Table 1. Main demographic and clinical characteristics of type 2 diabetes mellitus patients (n=328)

Variable	Value
Sex, male/female	243 (74)/85 (26)
Age, yr	65 (58–71)
Age at diagnosis of diabetes, yr	55 (49–61)
Diabetes duration, yr	9 (4–14)
Body mass index, kg/m^2	27 (25–29)
Waist circumference, cm	98 (92–103)
Female	95 (90–100)
Male	98 (93–104)
Steatosis grade (ultrasound)[a]	
Absent	17 (5)
Grade 1	153 (47)
Grade 2	96 (29)
Grade 3	60 (18)
Controlled attenuation parameter, dB/m[a]	248 (221–283)
<236	133 (40.8)
≥236–269	73 (22.4)
≥270–301	64 (19.6)
≥302	56 (17.2)
Liver stiffness, kPa	6.1 (4.9–8.6)
<5.9	147 (44.8)
≥5.9–7.9	87 (26.5)
≥7.9	94 (28.7)
Total cholesterol, mg/dL	171 (150–191)
HDL-C, mg/dL	46 (39–57)
Triglycerides, mg/dL	119 (87–168)
Aspartate aminotransferase, IU/L	18 (15–23)
Alanine aminotransferase, IU/L	21 (15–30)
Gamma glutamyltranspeptidase, IU/L	23 (16–36)
Platelet count, ×10^9/L	236 (201–283)
Fasting glucose, mg/dL	134 (113–163)
Albumin, g/L	40.4 (38.4–42.5)
HbA1c, mmol/mol	55 (48–64)
NAFLD score, unit	−0.515 (−1.263 to 0.246)
Significant fibrosis unlikely	61 (19)
Indeterminate	223 (68)
Significant fibrosis likely	44 (13)
FIB-4, unit	1.09 (0.86–1.47)
Significant fibrosis unlikely	218 (66)
Indeterminate	102 (31)
Significant fibrosis likely	8 (2)

Values are presented as number (%) or median (interquartile range).
HDL-C, high density lipoprotein cholesterol; HbA1c, glycosylated hemoglobin; NAFLD, nonalcoholic fatty liver disease; FIB-4, fibrosis-4.
[a]Data missing for two patients.

(*n*=51, 16%) or both (*n*=21, 6%) above the upper normal limit.

A CAP value indicative of steatosis (i.e., ≥236 dB/m) was detected in 195 of 328 patients (59.5%). Fifty-five of 133 patients, who did not show evidence of steatosis (41.3%), had LS values suggestive of fibrosis. Overall, the statistical association between CAP values suggestive of steatosis and LS values suggestive of fibrosis was highly significant (P<0.001).

Factors associated with significant fibrosis

Table 2 represents the univariate analysis of demographic, anthropometric, and clinical factors associated with significant fibrosis, defined as LS ≥7.9 kPa. The BMI was the strongest predictor of LS suggestive of significant fibrosis, while age at onset of diabetes and diabetes duration had no appreciable effect. The agreement between the interpretation of the LS value, lumped into three ordinal categories—based on LS values ≤5.9, >5.9 but <7.9, and ≥7.9 kPa—and the categories of significant fibrosis predicted by the NAFLD fibrosis score or those predicted by the Fib-4 was no better than that expected by chance (κ=−0.020, P=0.746; and κ=0.013, P=0.363, respectively). A better agreement was observed between NAFLD fibrosis score and Fib-4 (κ=0.106, P<0.001). This was however considered to be slight according to the Landis and Koch's scale [18].

Influence of genetic factors on NAFLD severity

Table 3 lists genotype and allele frequencies of the rs738409 (*PNPLA3*) single nucleotide polymorphism (SNP) and the insertion variant rs72613567 (*HSD17B13*) in the study population.

As shown in Fig. 1, the median LS increases from 5.9 kPa (4.7 to 7.7) in wild type (WT) C/C homozygotes to 6.1 kPa (5.2 to 8.7) in C/G heterozygotes and 6.8 kPa (5.8 to 9.2) in mutated (G/G) homozygotes. This trend was statistically significant (P=0.01). To investigate whether carriage of specific *HSD17B13* alleles modulated the aforementioned effect, we divided the population into two groups based on rs72613567, analyzed according to a dominant model. In WT homozygotes, the association between *PNPLA3* genotype and LS was fully maintained (C/C: 5.9 kPa [4.8 to 7.7]; C/G: 6.6 kPa [5.1 to 8.8]; G/G: 7.3 kPa [6.1 to 10.1]; P=0.008) (Fig. 2A). On the other hand, in the subgroup of patients carrying the *HSD17B13* variant allele the trend toward progressively higher LS disappeared completely (C/C: 5.7 kPa [4.6 to 7.7]; C/G: 5.4 kPa [5.2 to 8.6]; G/G: 5.4 kPa [4.8 to 5.8]; P=0.65) (Fig. 2B). In contrast to what observed for LS and *PNPLA3* genotype, the association between

Table 2. Univariate analysis of factors associated with significant liver fibrosis defined as liver stiffness ≥7.9 kPa

Variable	LS <7.9 kPa (*n*=234)	LS ≥7.9 kPa (*n*=94)	*P* value
Male sex	179 (24)	64 (68)	0.126
Age, yr	64 (58–71)	66 (58–71)	0.877
Age at diagnosis of diabetes, yr	54 (47–61)	56 (50–61)	0.132
Diabetes duration, yr	10 (5–15)	8 (3–12)	0.126
Body mass index, kg/m^2	26.6 (24.5–29.0)	28.0 (26.4–30.4)	<0.001
Waist circumference, cm	97 (90–101)	100 (95–107)	0.004
Female (*n*=85)	94 (90–99)	98 (90–103)	0.153
Male (*n*=179)	98 (92–103)	100 (95–107)	0.008
Steatosis grade (ultrasound)[a]			
Absent	14 (6)	3 (3)	
Grade 1	122 (53)	31 (33)	
Grade 2	62 (27)	34 (36)	
Grade 3	34 (15)	26 (28)	0.002
CAP, dB/m	242 (219–280)	266 (228–300)	0.022
Total cholesterol, mg/dL	170 (150–191)	172 (148–196)	0.865
HDL-C, mg/dL	47 (39–57)	44 (38–55)	0.171
Triglycerides, mg/dL	118 (81–153)	122 (98–194)	0.006
Aspartate aminotransferase, IU/L	17 (15–21)	22 (16–35)	0.001
>Upper normal limit	6 (3)	18 (19)	<0.001
Alanine aminotransferase, IU/L	20 (15–27)	25 (18–46)	<0.001
>Upper normal limit	22 (9)	29 (31)	<0.001
Gamma glutamyltranspeptidase, IU/L	21 (16–31)	30 (17–44)	0.001
>Upper normal limit	20 (9)	17 (18)	0.020
Platelet count, ×10^9/L	237 (201–284)	236 (201–284)	0.887
Fasting glucose, mg/dL	134 (113–160)	130 (109–168)	0.796
Albumin, g/L	40.4 (38.3–42.5)	40.4 (38.7–42.5)	0.913
HbA1c, mmol/mol	56 (48–65)	54 (48–60)	0.259
NAFLD score, unit	–0.531 (–1.351 to 0.271)	–0.447 (–0.861 to 0.092)	0.368
FIB-4, unit	1.07 (0.83–1.4)	1.18 (0.9–1.65)	0.048

Values are presented as number (%) or median (interquartile range).
LS, liver stiffness; CAP, controlled attenuation parameter; HDL-C, high density lipoprotein cholesterol; HbA1c, glycosylated hemoglobin; NAFLD, nonalcoholic fatty liver disease; FIB-4, fibrosis-4.
[a]Data missing for two patients.

CAP and the latter did not reach statistical significance. Indeed, the median CAP was 243.5 dB/m (219.0 to 277.0) in WT C/C homozygotes, 249.0 dB/m (221.0 to 284.0) in C/G heterozygotes, and 266.0 dB/m (224.0 to 308.0) in mutated (G/G) homozygotes (P=0.127). Moreover, HSD17B13 genotypes were clearly not related to CAP (P=0.806).

Interaction between genetic and environmental factors

To verify how genetic and environmental factors interact in determining the risk of severe NAFLD in T2DM, a multiple linear regression model was built to predict LS from age, gender, BMI, disease duration, HbA1c and *PNPLA3* genotype. A significant regression equation was found (F=5.07; df=6; R^2=0.087), indicating that both BMI and *PNPLA3* genotype were

Table 3. Genotype and allele frequencies of the genes of interest

PNPLA3 (n=328)		HSD17B13 (n=328)	
C/C	154 (0.47)	T/T	223 (0.68)
C/G	139 (0.42)	T/TA	90 (0.27)
G/G	35 (0.11)	TA/TA	15 (0.05)
G/*	174 (0.53)	TA/*	105 (0.32)
C	447 (0.68)	T	536 (0.82)
G	209 (0.32)	TA	120 (0.18)
HWE	0.66	HWE	0.14

The P values test the hypothesis of deviation from Hardy-Weinberg equilibrium (HWE).
PNPLA3, patatin like phospholipase domain containing 3; HSD17B13, 17β-hydroxysteroid-dehydrogenase type 13.

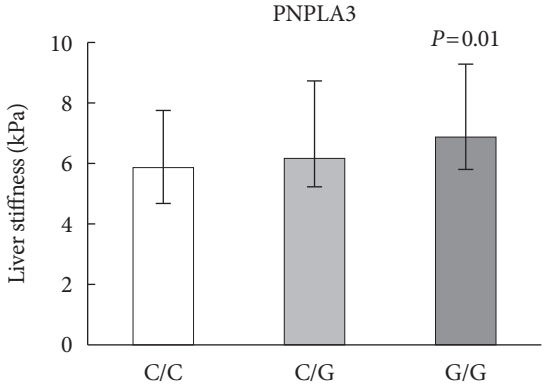

Fig. 1. Liver stiffness (kPa) distribution based on patatin like phospholipase domain containing 3 (PNPLA3) genotypes. Columns indicate medians, error bars interquartile ranges.

independent predictors of LS. Patients' predicted LS was equal to 1.023+0.028 (BMI in kg/m^2)+0.078 (*PNPLA3* genotype, coded 0, 1, or 2 according to the number of G alleles present). Based on this model, the predicted value of LS may vary from one reassuringly normal for patients with normal BMI who are rs738409 C/C homozygotes to another suggestive of progression to frank cirrhosis in obese patients who are rs738409 G/G homozygotes (Fig. 3).

Interestingly, in patients carrying at least one mutated allele in the *HSD17B13* gene, no predictor was identified according to the same linear regression model. Conversely, in WT *HSD17B13* subjects, the analysis replicated the findings obtained in the entire study population: F=5.36; df=6; R^2=0.130; LS=1.239+0.030 (BMI in kg/m^2)+0.085 (*PNPLA3* genotype, coded 0, 1 or, 2 according to the number of G alleles present).

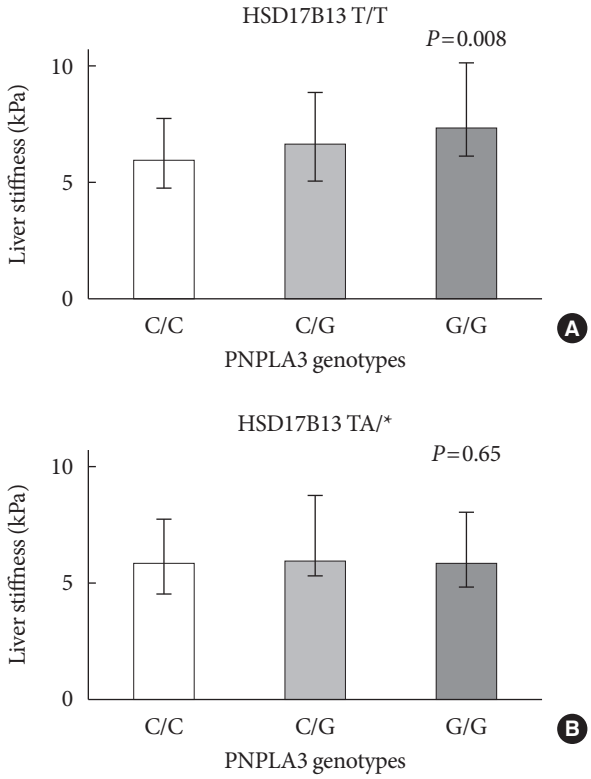

Fig. 2. Liver stiffness (kPa) distribution among patatin like phospholipase domain containing 3 (PNPLA3) genotypes among 17β-hydroxysteroid-dehydrogenase type 13 (HSD17B13) (A) wild type and (B) mutated carriers. Columns indicate medians, error bars interquartile ranges. *HSD17B13 T/T* (wild type), *HSD17B13 TA/** (heterozygous and/or homozygous "A" insertional mutation).

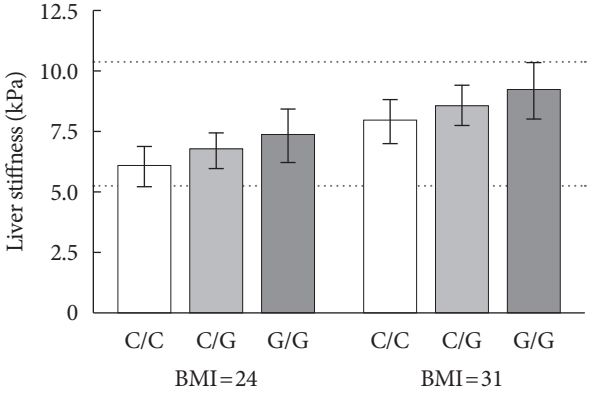

Fig. 3. Predicted values of liver stiffness (LS) (kPa), based on patatin like phospholipase domain containing 3 (*PNPLA3*) genotype and body mass index (BMI). Error bars indicate 95% confidence intervals. The upper horizontal dotted line indicates LS value suggestive of cirrhosis, the lower one the value suggestive of absent or minimal fibrosis.

DISCUSSION

In the present study, we show that—in an unselected cohort of non-morbidly obese T2DM patients—the prevalence of severe NAFLD is substantial. Anthropometric characteristics of patients explain most of the variability observed, with a significant contribution afforded by genes controlling lipogenesis and lipolysis. Remarkably, age and duration of T2DM appear not to contribute significantly to the prevalence of NAFLD. These findings and their possible clinical implications in the context of existing literature are discussed below.

The epidemic diffusion of obesity and T2DM, along with the improvement in HCV eradication thanks to antiviral agents, has made NAFLD the main cause of chronic liver disease and cirrhosis worldwide [19]. According to a recent projection, by 2030 the prevalence of NASH will have increased by 15% to 56%, while liver mortality and significant liver disease will have more than doubled as result of an aging/increasing population [20].

The natural history of NAFLD can vary significantly from patient to patient. Typically, NAFLD is a slowly progressive disease, with the vast majority of NAFLD patients never developing end-stage liver disease. The rate of progression is equal to one stage of fibrosis every 14 years in NAFLD, and every 7 years in NASH. However, in 20% of cases fibrosis progresses more rapidly [21]. Therefore, a significant proportion of NAFLD patients will experience liver-related complications, thus making an accurate risk prediction even more difficult.

In this context, T2DM patients are of major interest. First of all, the prevalence of NAFLD is higher in T2DM patients than in the general population [22]. Moreover, patients with established T2DM are more likely to suffer from more severe histological forms of NAFLD, even in cases where aminotransferase serum levels are normal [23], being aminotransferase levels an unreliable marker of NAFLD among patients with T2DM. Finally, the coexistence of NAFLD and T2DM typically worsens the course of both diseases [24].

However, despite the importance that should be given by clinicians to the diagnosis and treatment of NAFLD in T2DM patients, the usefulness of screening strategies to detect NAFLD has been recently questioned given our incomplete understanding of the natural history of this disease.

In our study, we highlight the essential contribution of excess body weight to liver fibrosis progression. In good agreement, a study on a cohort of 1,527 T2DM and NAFLD patients has recently shown that BMI is a predictor of FIB-4 score both at baseline and after 3 years of follow-up [25]. The reason why obesity negatively impacts liver functions is probably due to the fact that the adipose tissue, in obese subjects, acquires a proinflammatory, profibrogenic, and proangiogenic phenotype, resulting in abnormal production of adipokines and cytokines (e.g., leptin, interleukin [IL]-1, IL-6, and tumor necrosis factor-α [TNF-α]) involved in liver pathogenesis. For instance, leptin contributes to neoangiogenesis and directly modulates hepatic stellate cells in a profibrogenic manner [26]. Contextually, chemokines and proinflammatory cytokines, such as IL-1, IL-6, and TNF-α mediate macrophage infiltration, which causes adipose tissue "inflammation" in obese subjects, resulting in insulin resistance and deregulated secretion of adipokines [27]. Interestingly, obesity contributes not only to liver fibrosis progression but also to the complications brought by cirrhosis. In this regard, obesity is an independent risk factor for clinical cirrhosis decompensation, where dysregulated cytokines could contribute to worsening intrahepatic resistance and portal hypertension [28,29]. Moreover, obesity is a well known independent risk factor for the development of HCC [30]. Finally, hypovitaminosis D is highly prevalent in obese subjects due to the fact that vitamin D is a fat-soluble molecule stored in the adipose tissue [31]. In this regard, the severity of NAFLD has been inversely correlated with plasma vitamin D levels [32], possibly because vitamin D plays an antifibrogenic and anti-inflammatory role [33], similar to what observed in other inflammatory diseases [34].

Besides environmental factors and comorbidities, other genetic and epigenetic predictors of liver disease progression have been identified in recent years [35]. The strongest genetic predictor of chronic liver disease progression is the SNP rs738409 in *PNLPA3* gene. This gene encodes a membrane bound triacylglycerol lipase that mediates triacylglycerol hydrolysis. As *PNPLA3* mutation has been robustly associated to steatosis, fibrosis/cirrhosis, and HCC on a background of metabolic, alcoholic, and viral insults [36], in the present study we sought to determine whether *PNPLA3* mutation was associated with severity of liver disease. Previously, an increased risk of liver fibrosis was reported in T2DM patients carrying the mutated variant of rs738409 [37], although the estimation of liver fibrosis was based upon Fibrotest rather than on LS. From our data, we confirm the existence of a significant trend toward increasing LS in heterozygotes or mutated homozygotes over WT individuals. In particular, our results indicate that in

T2DM patients *PNLPA3* is associated with a significant risk of liver disease progression, although the strength of this association might be less relevant than in the general population probably because mainly driven by excess BMI and, by extension, body fat.

Another controversial point is that the association between *PNPLA3* and liver fibrosis does not automatically imply a causal relationship. However, the observation that insulin regulates *PNPLA3* gene expression [38] strongly argues in favor of a potential link between liver fibrosis progression and adiponutrin. Fittingly, adenovirus-mediated liver expression of a missense mutant form of PNPLA3 in mice was sufficient to abolish the hydrolase activity of adiponutrin leading to triglyceride accumulation, suggesting a possible cause-effect relationship between PNPLA3 polymorphism and development of steatosis and fibrosis [39]. Furthermore, in humans the missense mutation leads not only to intrahepatic remodeling but also to impairment of the fat efflux pathway from the liver, which seems to be caused by a reduction in very low density lipoproteins secretion [40].

It is also important to consider that *PNPLA3* is expressed by human hepatic stellate cells, where adiponutrin catalyzes the hydrolysis of retinyl esters [41]. Consistently, the missense mutation of *PNPLA3* gene is associated with lower circulating concentrations of free retinol [42] and higher intrahepatic concentrations of retinol [43], although whether this may contribute to fibrosis has yet to be defined.

Another important aspect further supporting a cause-effect link between PNPLA3 and liver fibrosis is that NAFLD patients carrying the variant rs738409 allele display prominent activation of the hepatic stem/progenitor cell niche, which is associated with a more aggressive histological pattern (i.e., portal fibrogenesis) and increased oxidative stress [44].

Although the association between *PNPLA3* gene and liver steatosis/fibrosis is well established, the pathogenetic mechanism underlying this association still remains unclear. In this regard, the detrimental impact of carrying the variant rs738409 allele has been recently shown to be mitigated by a concomitant mutation in the *HSD17B13* gene in the general population [3]. In this regard, one of the main findings of our study is that the *rs72613567:TA* mutation is associated with a lower risk of liver fibrosis progression also in case of T2DM, as shown for the general population. We find that the association between the variant rs738409 allele and severe NAFLD is fully maintained in WT *HSD17B13* carriers. By contrast, in those patients carrying at least one mutated allele, *PNPLA3* does not predict LS any longer. This suggests a potential protective role of such mutation, although the cross-sectional design of our study does not allow drawing definitive conclusions. In this regard, the reason why this mutation seems to play a protective role against liver diseases, mitigating the detrimental effect of the variant rs738409 allele, has yet to be determined. Su et al. [45] showed *HSD17B13* WT mRNA upregulation in NAFLD patients. Moreover, overexpression of *HSD17B13* in the livers of C57BL/6 mice and cultured hepatocyte cell lines enhanced substantially lipogenesis and increased the number and size of lipid droplets [45]. In the same study, upregulation of *HSD17B13* was observed in the livers of both diabetic mice and high-fat diet-fed mice, suggesting that *HSD17B13* may play a crucial role in the pathogenesis of fatty liver and may also be relevant in diabetes. Abul-Husn et al. [3] demonstrated in a large liver impairment patient cohort the important effect of HSD17B13 variant. The insertion of "A" in a splice donor site led to a protein-truncating isoform [3]. The same group, through RNA sequencing-based expression analysis demonstrated that *HSD17B13* variant was associated with decreased *PNPLA3* mRNA expression in an allele-dependent manner. Similarly, the 434K allele of *PNPLA3* could mitigate the effect of the *PNPLA3 I148M* allele on chronic liver disease by reducing hepatic *PNPLA3* mRNA and protein expression [46].

As expected, we found a strong correlation between steatosis development and T2DM, implying that the genetic component only exerts a marginal effect. This supports the hypothesis that, while fatty liver is a very common condition in T2DM patients, the development of liver fibrosis is significantly influenced by *PNPLA3*.

From a clinical standpoint, being obvious that major efforts should be devoted to correct excess body weight in T2DM, we provide further reasons to diabetologists to do so, by adding to the potential benefits of losing weight the reduced risk of progressive liver disease. Furthermore, our findings indicate that *PNLPA3* and *HSD13B17* products might represent potential therapeutic targets in the prevention of liver disease progression in T2DM patients. Though both variants might be worth of consideration in the construction of prediction models aimed to improve NAFLD detection in patients with T2DM, the inclusion of periodic TE examinations during patient evaluation would result in a cheaper and more direct screening protocol.

Among the several limitations of our study, we must ac-

knowledge the fact that TE is not the ideal method to characterize NAFLD. It is inferior to magnetic resonance based-techniques [15] and has not stood the test of time like liver biopsy. However, it is far more applicable than both techniques to clinical practice. Moreover, being a cross-sectional study we cannot provide date on the effect, if any, that prolonged inadequate control of T2DM might have on fibrosis progression, although punctual data at the time of our study appear not to point in that direction.

In conclusion, patients with T2DM have a high prevalence of liver disease characterized by significant fibrosis, which goes largely undetected in the absence of symptoms and striking laboratory abnormalities. Obesity and, to a minor but not negligible extent, genetic factors contribute to its development.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

AUTHOR CONTRIBUTIONS

Conception or design: M.B., C.C., M.N.B., L.S., R.C., V.R.M., L.M.C., G.S., G.P.C.S., R.M., M.P.
Acquisition, analysis, or interpretation of data: M.B., C.C., M.N.B., L.S., R.C., V.R.M., L.M.C., G.S., G.P.C.S., R.M., M.P.
Drafting the work or revising: M.B., C.C., M.P.
Final approval of the manuscript: M.B., C.C., M.N.B., L.S., R.C., V.R.M., L.M.C., G.S, G.P.C.S., R.M., M.P.

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