Association between nonalcoholic fatty liver disease and impaired cardiac sympathetic/parasympathetic balance in subjects with and without type 2 diabetes - The Cooperative Health Research in South Tyrol (CHRIS)-NAFLD Substudy

Giovanni Targher[1], Alessandro Mantovani[1], Christoph Grander[2], Luisa Foco[3], Benedetta Motta[3,4], Christopher D. Byrne[5,6], Peter Paul Pramstaller[3], Herbert Tilg[2]

[1]Section of Endocrinology, Diabetes and Metabolism, Department of Medicine, University and Azienda Ospedaliera Universitaria Integrata of Verona, Verona, Italy

[2]Department of Internal Medicine I, Gastroenterology, Hepatology, Endocrinology & Metabolism, Medical University Innsbruck, Innsbruck, Austria

[3]Institute for Biomedicine, Eurac Research (Affiliated to the University of Lübeck), Bolzano, Italy

[4]Department of Medicine and Surgery, University of Salerno, Salerno, Italy

[5]Nutrition and Metabolism, Faculty of Medicine, University of Southampton, Southampton, UK

[6]Southampton National Institute for Health Research Biomedical Research Centre, University Hospital Southampton, UK

Word count: abstract 250; text 4,275 (*excluding* title page, abstract, references, and tables); n. 3 **Tables** + n. 1 **Figure** + **online-only supplementary Material** (n. 1 supplementary Table).

Keywords: cardiac autonomic nervous system; CANS, nonalcoholic fatty liver disease; NAFLD; parasympathetic; sympathetic; type 2 diabetes

Address for correspondence:
Prof. Giovanni Targher, MD
Section of Endocrinology, Diabetes and Metabolism
Department of Medicine
University and Azienda Ospedaliera Universitaria Integrata
Piazzale Stefani, 1
37126 Verona, Italy
Phone: +39-045-8123110
E-mail: giovanni.targher@univr.it

ABSTRACT

Background and Aims: Cardiovascular disease (CVD) is the leading cause of death in patients with nonalcoholic fatty liver disease (NAFLD), both with, and without type 2 diabetes (T2DM). Cardiac autonomic dysfunction is a risk factor for CVD morbidity and mortality. The aim of this study was to assess whether there is an association between NAFLD and impaired cardiac autonomic function.

Methods and Results: Among the first 4,979 participants from the Cooperative Health Research in South Tyrol (CHRIS) study, we randomly recruited 173 individuals with T2DM and 183 age- and sex-matched nondiabetic controls. Participants underwent ultrasonography and vibration-controlled transient elastography (Fibroscan®, Echosens) to assess hepatic steatosis and liver stiffness. The low-frequency to high-frequency (LF/HF) power ratio and other heart rate variability measures were calculated from a 20-min resting electrocardiogram to derive a measure of cardiac sympathetic/parasympathetic imbalance. Among the 356 individuals recruited for the study, 117 had NAFLD and T2DM, 56 had T2DM alone, 68 had NAFLD alone, and 115 subjects had neither condition. Individuals with T2DM and NAFLD (adjusted-odds ratio 4.29, 95%CI 1.90-10.6) and individuals with NAFLD alone (adjusted-odds ratio 3.41, 95%CI 1.59-7.29), but not those with T2DM alone, had a substantially increased risk of having cardiac sympathetic/parasympathetic imbalance, compared to those without NAFLD and T2DM. Regression models were adjusted for age, sex, body mass index, hypertension, dyslipidemia, insulin resistance, hemoglobin A1c, C-reactive protein, and Fibroscan®-measured liver stiffness.

Conclusions: NAFLD was associated with cardiac sympathetic/parasympathetic imbalance, regardless of the presence or absence of T2DM, liver stiffness and other potential confounding factors.

LIST OF ABBREVIATIONS

ALT, alanine aminotransferase
AST, aspartate aminotransferase
ATC, Anatomical Therapeutic Chemical
BMI, body mass index
CANS, cardiac autonomic nervous system
CHRIS, Cooperative Health Research in South Tyrol
CRP, C-reactive protein
CVD, cardiovascular disease
ECG, electrocardiogram
e-GFR, estimated glomerular filtration rate
FFQ, Food Frequency Questionnaire
FIB-4, fibrosis-4
GGT, gamma-glutamyltransferase
HbA1c, hemoglobin A1c
HF, high-frequency
HOMA-IR, homoeostasis model assessment of insulin resistance
HRV, heart rate variability
LF/HF ratio, low-frequency to high-frequency ratio
NAFLD, non-alcoholic fatty liver disease
NASH, non-alcoholic steatohepatitis
pNN50, percentage of adjacent N-N intervals differing by more than 50 ms
RMSSD, square root of the mean squared difference of successive N-N intervals
SDANN, standard deviation of the average N-N interval in all minutes of the whole record
SDNN, standard deviation of normal-to-normal (N-N) intervals
SGLT2, sodium-glucose co-transporter-2
T2DM, type 2 diabetes
TP, total power
VCTE, vibration-controlled transient elastography
VLF, very low frequency

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease worldwide, affecting up to ~30% of the general adult population and up to ~70% of patients with type 2 diabetes mellitus (T2DM) [1, 2]. NAFLD includes a spectrum of pathologic liver conditions, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), advanced fibrosis and cirrhosis [3, 4]. Over the past decade, it has become increasingly clear that NAFLD is a "multi-system" disease [5], which is not only associated with increased risk of severe liver-related complications, but also with increased risk of cardiovascular disease (CVD) and other extra-hepatic diseases [6-8]. In this regard, it is now evident that CVD is the leading cause of mortality in patients with NAFLD [6, 7, 9].

Growing evidence indicates that dysfunction of the cardiac autonomic nervous system (CANS) is a risk factor for CVD mortality and morbidity [10, 11]. Given that CANS modulates heart rate and blood pressure, the presence of CANS dysfunction may predispose individuals to ischemic heart disease, arrhythmias and cardiac mortality [12-15], i.e., all cardiovascular outcomes that are often observed in people with NAFLD [6, 7, 9].

Several non-invasive techniques have been developed for the assessment of CANS function. The most widely used technique for assessing CANS function in routine clinical practice is measurement of heart rate variability (HRV), either spontaneously or after specific stimuli [16, 17]. Briefly, HRV mainly reflects the continuous interaction between the sinoatrial node rhythm and neural modulatory mechanisms and, hence, HRV measurement is a reliable, non-invasive test to assess CANS modulation *in vivo* [16, 17].

Presently, there are few and contrasting data regarding the association between NAFLD and impaired CANS function [18-20]. The precise mechanisms underpinning the association between NAFLD and increased CVD risk are poorly understood, but it is plausible that CANS dysfunction could be a mediator in the link between NAFLD and increased CVD risk. Consequently, is clinically relevant to determine whether CANS dysfunction may occur in individuals with NAFLD, and whether any association is independent of co-existing T2DM.

Thus, the aim of this observational study was to assess whether there is an association between NAFLD and impaired CANS function, as assessed by HRV measures, in individuals both with, and without T2DM, who were selected from the Cooperative Health Research in South Tyrol (CHRIS) study.

METHODS

Study design and recruitment of study participants

The present CHRIS-NAFLD sub-study is an observational study nested within the CHRIS study, which is an ongoing large population-based cohort study undertaken in South Tyrol, Italy. The design of the CHRIS study has been described elsewhere [21, 22]. Briefly, individuals who agreed to participate in the CHRIS study were invited to the study center at the Silandro Hospital (Bolzano, Italy) for receiving detailed medical interviews and visits, including a blood sample and urine collection, a 20-min 12-lead resting electrocardiogram (ECG) and other non-invasive clinical tests.

The CHRIS-NAFLD sub-study was designed after the recruitment of the first 4,979 CHRIS study participants, which was carried out between 2011 and 2014 (baseline data). From these 4,979 participants, we initially selected all individuals with established T2DM (n=227, i.e., cases) and an equal number of randomly selected nondiabetic control individuals (n=227), who were matched 1:1 for age and sex to the cases. This recruitment began in October 2016 and finished in February 2017. T2DM was defined according to the following diagnostic criteria [23] as: a positive response to the question "Has a doctor ever diagnosed you with diabetes?"; having fasting plasma glucose levels ≥126 mg/dl (≥7 mmol/L); a hemoglobin A1c (HbA1c) ≥6.5% (≥48 mmol/mol) or being treated with any antihyperglycemic agents. To prevent any misclassification of diabetes status in the control group, we also excluded individuals with prediabetes (defined as HbA1c level 5.7%-6.4% [39-47 mmol/mol]).

From the 454 initially selected matched cases and controls of the CHRIS-NAFLD sub-study, we subsequently excluded from statistical analysis individuals, who had significant alcohol intake (arbitrarily defined as alcohol consumption >30 g/day for men and >20 g/day for women) or those with a prior history of cancer, end-stage kidney disease, cirrhosis and/or chronic liver diseases due to viral hepatitis or hemochromatosis. As a consequence of these exclusion criteria, in this study we included a sample of 173 individuals with T2DM and 183 age- and sex-matched nondiabetic controls without significant alcohol consumption and known liver diseases. More details about the protocol and recruitment of the CHRIS-NAFLD sub-study have been published elsewhere [24].

The CHRIS-NAFLD sub-study was approved by the Ethical Committee of the Healthcare System of the Autonomous Province of Bolzano (Südtiroler Sanitätsbetrieb/Azienda Sanitaria dell'Alto

Adige), protocol n. 85-2016. A written informed consent was obtained from each participant before the study enrolment.

Clinical and laboratory data

All participants of the CHRIS-NAFLD sub-study underwent an interview involving questions about their state of health, anthropometric and biochemical measurements, as well as ultrasound and transient elastography examinations to evaluate the presence of hepatic steatosis and liver stiffness (as specified below). Body mass index (BMI) was calculated by dividing weight (in kilograms) by height (in square meters). Waist circumference was measured at the midpoint between the lowest rib and the iliac crest, according to the World Health Organization protocol [25]. Blood pressure was measured with a mercury manometer at the right upper arm using an appropriate cuff size after the subject had been seated quietly for at least 5 min.

Questionnaires concerning the change of participants' health status since the baseline participation and their life-style habits were administered by an interviewer, and the use of medications in the last week was documented using an electronic optical scan of their medication box bar codes, according to the Anatomical Therapeutic Chemical (ATC) classification system [24]. A Food Frequency Questionnaire (FFQ) based on the Global Allergy and Asthma European Network of Excellence study [26] was mailed to their homes prior to participation, in order to limit the time spent at the study center. The FFQ also asked about the average frequency of the consumption of alcoholic drinks over the last year (rarely or never, 1-3/month, 1/week, 2-4/week, 5-6/week, 1/day, 2+/day), specifically of beer (200 mL), red wine (125 mL), white wine (125 mL), rosé wine (125 mL), liqueurs (50 mL) or spirits (50 mL). Participants also answered an interviewer-

administered questionnaire on smoking status, based on the European Community Respiratory Health Survey [27] from which we also derived pack-years as a measure of cumulative smoking.

Hypertension was present if reported from the interview or if blood pressure was ≥140/90 mmHg or in presence of any anti-hypertensive treatment (corresponding to ATC codes starting with C02, C03, C04, C07, C08, C09) [24]. Dyslipidemia was present if reported from the interview, or if serum total cholesterol levels was >200 mg/dL (>5.1 mmol/L) or in presence of any lipid-lowering treatment. Presence of ischemic heart disease was defined as a documented history of myocardial infarction, angina, coronary revascularization procedures or typical electrocardiographic abnormalities.

Serum liver enzymes (aspartate aminotransferase [AST], alanine aminotransferase [ALT], and gamma-glutamyltransferase [GGT]), glucose, HbA1c, lipids (total cholesterol, HDL-cholesterol, triglycerides), creatinine, electrolytes, C-reactive protein (CRP), insulin and other biochemical blood measurements were centrally measured in all participants after an overnight fast using standard laboratory procedures described elsewhere [21, 22]. LDL-cholesterol was calculated using the Friedewald's equation. The homoeostasis model assessment of insulin resistance (HOMA-IR) score was used for estimating insulin resistance. Glomerular filtration rate (e-GFR) was estimated by using the Chronic Kidney Disease-Epidemiology Collaboration (CKD-EPI) study equation [28]. We also calculated the fibrosis-4 (FIB-4) index by using the following formula: age × AST (IU/L)/platelet count ($\times 10^9$/L) × √ALT (IU/L) [29].

Assessment of hepatic steatosis and fibrosis

All participants underwent both liver ultrasonography (5-1 MHz Phased Array Transducer, iViz, SonoSite, USA) and vibration-controlled transient elastography (VCTE; Fibroscan®, Echosens, France) that were performed by a single trained medical doctor, who was blinded to all participants' clinical and laboratory details.

Diagnosis of hepatic steatosis was based on standard sonographic features [30]. Hepatic steatosis was classified into three grades: normal or very slight increase in the echo pattern with normal visualization of vessels and diaphragm (grade 1); moderate increase in echogenicity with reduced visibility of portal vein and diaphragm (grade 2); and distinct increase in echo pattern with poor visibility of intrahepatic vessels and diaphragm (grade 3). NAFLD was defined as evidence of hepatic steatosis on ultrasonography, after exclusion of excessive alcohol intake and other competing causes for hepatic steatosis, such as viral hepatitis, hereditary liver disorders or other chronic liver diseases [3, 4].

To perform VCTE (Fibroscan®) participants were placed in a supine position with their right arm fully adducted and asked to hold their breath. At least ten independent VCTE measurements were taken, using either a M+ probe or a XL+ probe according to participant's body weight [31, 32]. VCTE values were defined as unreliable when the interquartile range (IQR) to median ratio was >30%. Hepatic fibrosis was graded into four stages, from F0 to F4, based on VCTE-derived measurements of liver stiffness (LSM) [33]. LSM values of ≥7.0 kPa were used for diagnosing clinically significant fibrosis (that corresponds to stage ≥F1 on liver histology) [34].

HRV measures

In all participants of the CHRIS-NAFLD sub-study, the measurements of HRV were performed only between 2011 and 2014 (and were not repeated in 2016). Specifically, the HRV measures were derived from a 20-min 12-lead resting ECG, obtained using a PC-ECG-System Custo 200 (Customed) workstation with a sampling rate of 1,000 Hz, as detailed previously [35]. Participants were asked to remain in supine position and silent during the ECG examination. We analyzed the following time-domain measures of HRV: R-R intervals (or Normal to Normal [N-N] intervals), standard deviation of normal-to-normal (N-N) intervals (SDNN), standard deviation of the average N-N interval in all minutes of the whole record (SDANN), square root of the mean squared difference of successive N-N intervals (RMSSD), and the percentage of adjacent N-N intervals differing by more than 50 ms (pNN50). RMSSD and pNN50 are mainly associated with high-frequency power (HF) and, therefore, parasympathetic activity, whereas SDNN is mainly associated with low-frequency power (LF), and, hence, sympathetic activity [36]. We also measured the following frequency-domain measures of HRV: total power, which corresponds to the area under the spectral curve from 0 to 0.4 Hz and is a general index of HRV; VLF, power in the very-low-frequency band from 0.003 to 0.04 Hz; LF, power in the low-frequency band, from 0.04 to 0.15 Hz, reflecting predominantly the cardiac sympathetic activity; HF, power in the high-frequency band, from 0.15 to 0.4 Hz, reflecting predominantly the cardiac parasympathetic (vagal) activity; and the low-frequency to high-frequency power (LF/HF) ratio, which reflects the sympatho-vagal interaction and, hence, the cardiac sympathetic/parasympathetic balance [36], and which, if altered, is strongly associated with an increased risk of CVD morbidity and mortality [12-15]. We were not able to perform transthoracic echocardiographic examinations.

Statistical analysis

Data are expressed as means and SD for normally distributed continuous variables, or as medians and interquartile ranges (p25-p75) for not normally distributed variables, or as percentages for categorical variables. Differences among the four subgroups of individuals (i.e., persons with T2DM and NAFLD, persons with T2DM alone, persons with NAFLD alone, and persons with neither condition) were tested by the one-way ANOVA for normally distributed variables, the Kruskal-Wallis test for not normally distributed variables or the chi-squared test for categorical variables. A binary logistic regression analysis was used to examine the association between NAFLD and/or T2DM status and presence of cardiac sympathetic/parasympathetic imbalance, defined as increased LF/HF ratio (i.e., the dependent variable, which was included as 3rd tertile of LF/HF ratio *vs*. 1st and 2nd tertiles combined). The following three forced-entry multivariable logistic regression models were performed: an unadjusted model; a model adjusted for age and sex (model 1); and finally, a model further adjusted for BMI, hypertension (defined as blood pressure ≥140/90 mmHg or use of any anti-hypertensive treatment, including beta-blockers), dyslipidemia, HbA1c, HOMA-IR score, plasma CRP levels or VCTE-measured liver stiffness (model 2). We did not additionally adjust also for heart rate, because the LF/HF ratio is a frequency-domain measure of HRV. Covariates included in these multivariable logistic regression models were chosen as potential confounding factors based on their significance in univariate analyses or based on their biological plausibility. The likelihood ratio test was used to evaluate the difference between these regression models. In addition, we also performed a multivariable linear regression analysis to test the independent association between presence of NAFLD and/or T2DM and increasing values of the LF/HF ratio that was logarithmically transformed before the statistical analysis and then included as a continuous measure (log LF/HF ratio). Covariates included in these multivariable linear regression models were the same of those included in the aforementioned multivariable logistic

regression models. A *p*-value <0.05 was considered as statistically significant. STATA 16.1 (Stata Corporation, College Station, Texas, USA) was used to perform all statistical analyses.

RESULTS

In the CHRIS-NAFLD sub-study, 173 individuals with known T2DM and 183 age- and sex-matched nondiabetic controls were studied. Among these 356 participants [M/F=179/177; median age 68 (p25-p75: 61-75) years; median BMI 27.6 (24.6-30.9) kg/m^2], who did not have significant alcohol consumption and known liver diseases, 117 (32.9%) had both NAFLD and T2DM, 68 (19.1%) had NAFLD alone, 56 (15.7%) had T2DM alone, and 115 (32.3%) subjects had neither condition. In total, 90 out of 173 (52%) individuals with established T2DM were treated with antihyperglycemic drugs. Most of them (n=73) were treated with metformin alone, or in combination with either sulphonylureas/glinides (n=12), or incretin-based drugs (mostly dipeptidylpeptidase-4 inhibitors). Only a minority of T2DM subjects assumed insulin (n=9), pioglitazone (n=3) or sodium-glucose co-transporter-2 (SGLT2) inhibitors (n=2).

Table 1 shows that the main clinical and biochemical characteristics of participants, stratified by NAFLD and T2DM status. Individuals with T2DM and NAFLD were younger and were more likely to be centrally obese, hypertensive and insulin resistant (as reflected by higher fasting insulin concentrations and greater HOMA-IR score), and more likely to have higher HbA1c, higher plasma triglycerides and lower HDL-cholesterol levels compared to the other groups. Individuals with T2DM and NAFLD also had higher circulating levels of CRP, liver enzymes, as well as higher FIB4 score and greater VCTE-measured liver stiffness (including a higher proportion of persons with VCTE-measured LSM $\geq$7 or $\geq$8 kPa, suggestive of significant/advanced fibrosis). Sex, smoking

history, prior ischemic heart disease and/or stroke, electrolytes and current use of any glucose-lowering agents did not differ significantly among the four groups. However, looking at the specific classes of glucose-lowering agents, individuals with T2DM and NAFLD were more likely to be treated with insulin and less likely to be treated with metformin, compared to subjects with T2DM but without NAFLD. No significant differences were found in the current use of sulphonylureas/glinides, pioglitazone, SGLT2-inhibitors or incretin-based drugs. The use of statins and anti-hypertensive agents (mostly renin-angiotensin system inhibitors, calcium-channel blockers or diuretics) was more frequent among T2DM patients with, or without NAFLD, compared to the other two groups. A negligible proportion (~1% of total) of these subjects were taking beta-blockers. None of them were treated with antiarrhythmic drugs.

Table 2 shows the time-domain and frequency-domain measures of HRV in participants stratified by NAFLD and T2DM status. Individuals with T2DM and NAFLD had significantly higher values of heart rate and LF/HF ratio, lower RMSSD and marginally lower HF compared to the other groups of individuals, thereby suggesting a decreased cardiac parasympathetic activity. Conversely, no significant differences were observed in SDNN, SDANN, pNN50, as well as total power, VLF and LF component among the four groups of individuals.

Figure 1 shows the boxplot of the continuous values of LF/HF ratio (panel A), as well as the proportion of subjects with a high LF/HF ratio (i.e., arbitrarily defined as subjects belonging to the 3rd tertile of distribution of the LF/HF ratio; panel B) among participants stratified by NAFLD and T2DM status. In both analyses, the LF/HF ratio (included as either a continuous or categorical variable) was significantly higher in individuals with T2DM and NAFLD than in the other three groups. It is reasonable to assume that the increase of the LF/HF ratio we observed in individuals

with NAFLD was mainly dependent on a reduction of the HF component rather than an increase of the LF component, as specifically shown in **Table 2.**

Table 3 shows the association between NAFLD and/or T2DM status and presence of cardiac sympathetic/parasympathetic imbalance, as defined by a high LF/HF ratio (3rd tertile *vs*. 2nd and 1st tertiles combined). In univariable regression analysis, individuals with T2DM and NAFLD (unadjusted-odds ratio [OR] 3.48, 95% CI 1.92-6.30) and individuals with NAFLD alone (unadjusted-OR 2.63, 95% CI 1.32-5.32), but not those with T2DM alone, had a substantially increased risk of cardiac sympathetic/parasympathetic imbalance compared to those without NAFLD or without T2DM (control group). These results remained essentially unchanged after adjustment for age and sex (adjusted model 1), or even after additional adjustment for BMI, hypertension (defined as blood pressure $\geq$140/90 mmHg or use of any anti-hypertensive agents, including beta-blockers), dyslipidemia, HbA1c, HOMA-IR score, plasma CRP levels or VCTE-measured liver stiffness (model 2). In this latter regression model, female sex was the only other variable that was independently associated with a lower risk of cardiac sympathetic/parasympathetic imbalance. Conversely, VCTE-measured liver stiffness was not independently associated with risk of cardiac sympathetic/parasympathetic imbalance. Almost identical results were found when we excluded patients with prior ischemic heart disease or stroke (n=5) from the aforementioned regression model 2 (data not shown).

Supplementary Table S1 shows the results of multivariable linear regression analyses adjusting for the same list of covariates mentioned above, and where the values of the LF/HF ratio were included as a continuous variable (i.e., logarithmically transformed LF/HF ratio) in these regression models. In univariable regression analysis, individuals with both T2DM and NAFLD and individuals

with NAFLD alone, but not those with T2DM alone, had significantly higher LF/HF ratio values than those without NAFLD or without T2DM (control group). These results did not change after adjusting for traditional cardiovascular risk factors, HbA1c, HOMA-IR, plasma CRP levels or VCTE-measured liver stiffness (model 2).

DISCUSSION

The main and novel findings of our observational pilot study are that the presence of imaging-defined NAFLD, regardless of the presence or absence of T2DM, was significantly associated with an impaired cardiac sympathetic/parasympathetic balance. Impaired cardiac sympathetic/parasympathetic balance was manifest as a high LF/HF ratio, suggesting a decrease in parasympathetic (vagal) activity. Notably, the significant association between NAFLD and cardiac autonomic imbalance persisted after adjustment for age, sex, adiposity measures, hypertension, dyslipidemia, HOMA-IR score, HbA1c, plasma CRP levels or VCTE-measured liver stiffness.

To date, there are limited, but conflicting data regarding the association between NAFLD and CANS dysfunction, as assessed by HRV measures [18-20]. In a cross-sectional study involving 497 Taiwanese individuals enrolled during a health checkup program (35% of which had NAFLD), Liu *et al*. reported that some HRV measures (especially SDNN), as detected by 5-min 12-lead resting ECGs, were decreased in subjects with NAFLD compared to those without NAFLD [18]. In another small study involving 75 Indian individuals (25 with both NAFLD and T2DM, 25 with NAFLD alone, and 25 controls), Kumar *et al.* showed that the LF/HF ratio, detected on 5-min 12-lead ECGs, did not significantly differ among the three groups [19]. In a study including 46 UK patients with

NAFLD, 16 patients with hepatic steatosis and excessive alcohol intake (i.e., having a dual aetiology fatty liver disease, DAFLD) and 34 control subjects, Houghton *et al.* reported that both individuals with NAFLD and those with DAFLD had impaired CANS function (assessed by HRV measures using power spectral analysis) when compared to control subjects [20]. Similarly, in a study of 189 subjects with biopsy-proven NAFLD, it has been shown that dysregulated neurovascular control underlies declining microvascular functionality in adults with NAFLD, suggesting a mechanistic role for dysregulated neurovascular control of the vasculature in NAFLD [37]. Some small studies have also reported the presence of impaired heart recovery index after exercise (treadmill test) in individuals with NAFLD [38, 39].

It is known that CANS dysfunction plays an important role in left ventricular remodeling, ventricular tachyarrhythmias and sudden cardiac death [12-15]. For that reason, we believe that the findings of our study are clinically relevant, because they may contribute to better explain the increased risk of CVD morbidity and mortality observed in people with NAFLD [6, 7, 9]. Furthermore, a better understanding of the pathophysiology of CANS dysfunction in NAFLD patients with, and without T2DM, might also contribute to the discovery of novel therapeutic interventions for reducing CVD risk associated with NAFLD.

To date, a clear understanding of the precise mechanisms linking NAFLD to impaired CANS function remains elusive. The most obvious explanation for our findings is that NAFLD is associated with CANS dysfunction simply as a consequence of the shared cardiometabolic risk factors (for example, obesity, insulin resistance, T2DM, or low-grade chronic inflammation, which are also known risk factors for impaired CANS function [40-42]). However, it should be noted that in our study NAFLD was associated with cardiac sympathetic/parasympathetic imbalance, independent

of T2DM status, and other shared cardiometabolic risk factors (such as BMI, hypertension, dyslipidemia, HOMA-IR score, and plasma CRP levels). In addition, this association remained significant even after further adjustment for VCTE-measured liver stiffness. This finding suggests that additional NAFLD-related mechanisms, beyond shared cardiometabolic risk factors, might be involved in the link between NAFLD and cardiac sympathetic/parasympathetic imbalance. It is possible to speculate that NAFLD, especially in its more advanced histologic forms, might at least in part contribute to the development and progression of CANS dysfunction (mainly parasympathetic dysfunction), possibly through the exacerbation of hepatic/systemic insulin resistance and atherogenic dyslipidemia, as well as the release of a variety of proinflammatory cytokines, prooxidant factors and profibrogenic mediators that promote nerve damage in the long term [43, 44]. However, more mechanistic studies are needed to investigate this speculation. Our individuals with known T2DM were randomly recruited from the general population and had good glycemic control. Thus, these results cannot necessarily be extrapolated to the general population of patients with T2DM, particularly those with poor glycemic control and/or more complicated disease. On the other hand, this limitation also highlights an interesting and potentially important observation; namely that NAFLD may be associated with cardiac sympathetic/parasympathetic imbalance, in the absence of major changes in glycaemia.

The most important limitation of our observational study is its cross-sectional design that precludes us establishing any causal and temporal relationship between NAFLD and CANS dysfunction. Other limitations that should be mentioned are the lack of any echocardiographic data and the use of liver ultrasonography for diagnosing hepatic steatosis (that is relatively insensitive to the presence of low amounts of hepatic fat) and Fibroscan® for staging liver fibrosis. These two imaging techniques are widely used in clinical practice for diagnosing and staging

NAFLD. However, we did not perform neither liver biopsies, which would be unethical to perform in our individuals who had normal or only slightly elevated serum liver enzymes, nor magnetic resonance-based imaging techniques (including magnetic resonance elastography), which are not easily accessible for population-based cohort studies. Another important limitation of our study is that HRV measurements were undertaken between 2011 and 2014 and liver imaging techniques were performed in 2016 (after a median period of ~3 years). Although in 2016 we examined only individuals with T2DM and nondiabetic controls without significant alcohol consumption and known liver diseases, it is possible that this time difference may have contributed to an underestimation of NAFLD at baseline. However, given the chronic, indolent disease courses of both NAFLD and CANS dysfunction, we believe that this time discrepancy is unlikely to have had a significant effect on the association between NAFLD and impaired CANS function. In fact, although there are very few prospective studies examining NAFLD incidence rates (none of which published in European populations), a meta-analysis estimated that the pooled regional NAFLD incidence rate estimates for Israel and Asia (which are among the countries with the highest prevalence of NAFLD) were ~2.8 and ~5.0 per 100 person-years, respectively [1]. Thus, although some non-differential misclassification of NAFLD on the basis of liver ultrasonography is likely (i.e., some persons could have underlying NAFLD despite negative ultrasonography examination), this limitation would serve to attenuate the magnitude of our effect measures toward null; thus, our results can probably be considered to be conservative estimates of the association between NAFLD and impaired CANS function. That said, additional studies with contemporaneous liver imaging techniques and HRV assessments are required to confirm our findings. Although we adjusted our results for a comprehensive range of potential confounders, we cannot exclude the possibility of residual confounding or confounding by unmeasured or unknown factors. Finally, the

CHRIS participants are of European ancestry and thus we are unable to comment on the generalizability of our findings to other non-Caucasian ethnic groups.

Despite these limitations, our study has important strengths, including the relatively large sample size that was randomly recruited from a well-phenotyped, community-based cohort of individuals, the completeness of the dataset, the use of validated techniques to measure HRV (including the use of the LF/HF ratio, which is a widely used index reflecting the balance of cardiac autonomic nervous system), the ability to adjust for established cardiometabolic risk factors, and the exclusion of patients with significant alcohol consumption and important comorbid conditions, such as end-stage renal disease, malignancies or cirrhosis.

In conclusion, the findings of our observational study suggest that imaging-defined NAFLD is significantly associated with cardiac sympathetic/parasympathetic imbalance, regardless of the presence T2DM, established cardiometabolic risk factors and VCTE-measured liver stiffness. These findings may also inform understanding of the increased CVD morbidity and mortality observed in patients with NAFLD. However, we suggest that further studies are needed to better decipher the existing but complex links between NAFLD and impaired cardiac autonomic function.

ACKNOWLEDGMENTS

The CHRIS study is a collaborative effort between the Eurac Research Institute for Biomedicine and the Healthcare System of the Autonomous Province of Bozen/Bolzano (Südtiroler Sanitätsbetrieb/Azienda Sanitaria dell'Alto Adige). Investigators thank all study participants from the middle and upper Vinschgau/Val Venosta, the general practitioners, and the personnel of the

Hospital of Schlanders/Silandro for their support and collaboration. The 20-min electrocardiographic traces were processed at the Technical University of Munich by the Biosignal Analysis Working Group (director: Dr. Georg Schmidt). We thank the Labormed Srl – GmbH, Bolzano (represented by Dr. Monica Cembran) for funding the insulin measurements, which were performed by Synlab Italia Srl, Castenedolo. Extensive acknowledgement is reported at https://translational-medicine.biomedcentral.com/articles/10.1186/s12967-015-0704-9. The CHRIS study is funded by the Department of Innovation, Research and University of the Autonomous Province of Bozen/Bolzano. GT is supported in part by grants from the University School of Medicine of Verona, Verona, Italy. CDB is supported in part by the Southampton NIHR Biomedical Research Centre (IS-BRC-20004), UK.

AUTHORS CONTRIBUTIONS

Conceptualization, Methodology, Software, Resources and Supervision: Peter Paul Pramstaller; Investigation, Validation and Data Curation: Christoph Grander, Luisa Foco and Benedetta Motta; Formal Statistical Analysis: Alessandro Mantovani and Giovanni Targher; Writing- Original draft preparation: Giovanni Targher; Writing- Reviewing and Editing: All authors.

CONFLICTS OF INTEREST: nothing to declare.

Table 1. Main clinical and biochemical characteristics of participants, stratified by NAFLD and type 2 diabetes (T2DM) status.

Variables	**Without NAFLD/Without T2DM (n=115)**	**With T2DM/Without NAFLD (n=56)**	**With NAFLD/Without T2DM (n=68)**	**With NAFLD/With T2DM (n=117)**	***P* values**
Age (years)	67 (58-76)	73 (65.5-79)	70 (64-75)	66 (58-71)	<0.001
Sex (M/F)	55/60	25/31	35/33	64/53	0.585
BMI (kg/m^2)	24.8 (22.9-27.2)	26.3 (23.8-28.5)	28.3 (25.7-30.6)	31.2 (28.2-34.1)	<0.001
Waist circumference (cm)	85 (77-93)	91 (84-97)	97 (88-103)	104 (97-111)	<0.001
Systolic blood pressure (mmHg)	134 (124-145)	139 (124-160)	138 (129-148)	142 (129-152)	0.047
Diastolic blood pressure (mmHg)	82 ± 9	83 ± 10	85 ± 9	87 ± 9	<0.01
Alcohol intake (g/day)	4.80 (0.96-13.50)	1.91 (0.07-12.71)	4.90 (1.02-19.97)	2.79 (0-15.07)	0.179
Current smokers (%)	4.3	8.9	7.3	6.9	0.646
Hypertension (%)	56.5	78.6	76.5	87.2	<0.001
Dyslipidemia (%)	80.9	87.5	80.9	82.1	0.729
Fasting glucose (mmol/l)	4.94 (4.67-5.28)	6.17 (5.61-7.44)	5.11 (4.86-5.39)	7.03 (6.28-8.06)	<0.001
Fasting insulin (mUI/l)	4.7 (3.6-6.6)	6.1 (4.55-9.45)	8.3 (5.7-11.7)	12.6 (8.5-17.4)	<0.001
HOMA-IR score	1.03 (0.79-1.49)	1.63 (1.22-2.65)	1.96 (1.25-2.56)	3.93 (2.67-5.97)	<0.001
Total cholesterol (mg/dl)	215 ± 43	204 ± 47	218 ± 51	197 ± 42	0.006
LDL-cholesterol (mg/dl)	134 (104-161)	125 (96-151)	138 (114-168)	122 (94-144)	0.040
HDL-cholesterol (mg/dl)	58 (51-70)	56 (47.5-65)	52 (45.5-64.5)	50.5 (41.5-59)	<0.001
Triglycerides (mg/dl)	79 (65-112)	97 (79-117)	108.5 (79.5-136)	117 (95-158.5)	<0.001
HbA1c (%)	5.3 (5.2-5.4)	6.2 (5.8-6.6)	5.3 (5.2-5.4)	6.4 (6.1-7.0)	<0.001
Creatinine (mg/dl)	0.82 (0.76-0.96)	0.84 (0.77-0.96)	0.84 (0.78-0.96)	0.85 (0.78-0.98)	0.785
e-GFR$_{CKD-EPI}$ (ml/min/1.73 m^2)	82 (73-89)	78 (69-86)	77 (71-86)	82 (73-91)	0.063
FIB4 score	1.43 (1.11-1.95)	1.46 (1.05-1.9)	1.37 (1.14-1.79)	1.17 (0.85-1.48)	<0.001
Prior ischemic heart disease or stroke (%)	1.7	1.8	0	2.6	0.403
ALT (UI/l)	16 (12-22)	15 (12-19)	20 (16-26)	23 (18-28)	<0.001
AST (UI/l)	20 (17-22)	18 (16-21)	21 (18-24)	20 (16-25)	<0.001
GGT (UI/l)	19 (15-28)	19 (15-28)	27 (19-37)	32 (22-52)	<0.001
C-reactive protein (mg/dl)	0.12 (0.08-0.22)	0.16 (0.09-0.27)	0.20 (0.11-0.34)	0.23 (0.12-0.4)	<0.001
Sodium (mmol/l)	141 (139-142)	140 (139-142)	141 (140-142)	140 (139-142)	0.426
Potassium (mmol/l)	4.6 (4.3-4.8)	4.7 (4.4-5)	4.5 (4.3-4.8)	4.6 (4.4-4.8)	0.254
Fibroscan®-assessed LSM (kPa)	3.8 (3.1-4.7)	4.1 (3.3-5.1)	4.3 (3.8-5.6)	5.1 (4-6.1)	<0.001
LSM ≥7 kPa (%)	0.9	8.9	13.2	19.7	<0.001
LSM ≥8 kPa (%)	0	5.4	10.3	16.2	<0.001
Lipid-lowering drugs (%)	17.0	44.6	25.0	45.6	<0.001
Anti-hypertensive drugs (%)	30.4	57.1	42.7	67.5	<0.001
Glucose-lowering drugs (%)	NA	44.6	NA	55.3	0.193*

Variables	**Without NAFLD/Without T2DM (n=115)**	**With T2DM/Without NAFLD (n=56)**	**With NAFLD/Without T2DM (n=68)**	**With NAFLD/With T2DM (n=117)**	***P* values**
Metformin (%)	NA	96.0	NA	76.2	0.024*
Sulphonylureas/glinides (%)	NA	16.0	NA	22.2	0.770*
Incretin-based agents (%)	NA	12.0	NA	20.6	0.541*
Pioglitazone or SGLT-2 inhibitors	NA	8.0	NA	5.0	0.620*
Insulin (%)	NA	0	NA	14.3	0.041*

Sample size, *n*=356. Data are reported as means ± SD or medians and interquartile range (p25-p75). Differences among the groups of participants were tested by the one-way ANOVA for normally distributed continuous variables (i.e., cholesterol levels and diastolic blood pressure), the Kruskal-Wallis test for not normally distributed continuous variables or the chi-squared for categorical variables.
*In this case, the chi-square test or the Fisher exact test (when appropriate) compares glucose-lowering drug intakes only amongst those with T2DM.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BP, blood pressure; GGT, gamma-glutamyltransferase; HOMA-IR, homeostatic model assessment of insulin resistance; eGFR, estimated glomerular filtration rate; FIB-4, fibrosis-4; SGLT-2, sodium-glucose cotransporter-2; LSM, liver stiffness measurement; NA, not available.

Table 2. Heart Rate Variability measures in participants, stratified by NAFLD and type 2 diabetes (T2DM) status.

Variables	**Without NAFLD/Without T2DM (n=115)**	**With T2DM/Without NAFLD (n=56)**	**With NAFLD/Without T2DM (n=68)**	**With NAFLD/With T2DM (n=117)**	***P* values**
HR (bpm)	60 (55-66)	63 (58-69)	62 (57-67)	64 (58-70)	0.006
SDNN (ms)	42 (34-56)	39 (29-54)	45 (34-55)	40 (31-49)	0.080
SDANN (ms)	11 (7.4-16)	9.1 (5.4-15)	12.0 (6.5-16)	9.8 (5.7-15)	0.109
RMSSD (ms)	26 (19-36)	27 (16-41)	25 (18-36)	23 (15-30)	0.022
pNN50	5.2 (2.2-17)	5.3 (1.5-20)	4.0 (1.4-14)	4.0 (0.88-11)	0.238
TP (ms^2)	1,453 (903-2,340)	1,162 (689-2,525)	1,608 (777-2,231)	1,216 (690-1,997)	0.214
VLF (ms^2)	677 (455-995)	532 (277-881)	645 (398-1018)	532 (334-884)	0.125
LF (ms^2)	322 (176-592)	284 (111-534)	318 (158-578)	303 (160-582)	0.773
HF (ms^2)	131 (80-251)	129 (41-410)	116 (50-275)	94 (41-209)	0.071
LF/HF ratio	2.20 (1.33-3.09)	2.00 (1.04-3.77)	2.68 (1.60-4.66)	3.09 (1.68-5.20)	0.002

Sample size, *n*=356. Data are reported as medians and interquartile range (p25-p75). Differences among the four groups of participants were tested by the Kruskal-Wallis test.

Abbreviations: HR, heart rate, pNN50, percentage of interval differences of successive N-N intervals greater than 50 ms; RMSSD, root mean square of successive differences between adjacent N-N intervals; SDANN, standard Deviation of the average N-N interval in all minutes of the whole record; SDNN, time domain with the standard deviation of N-N intervals; TP, total power, VLF, very low frequency; LF, low frequency; HF, high frequency.

Table 3. Logistic regression analyses – Association between NAFLD and/or T2DM status and presence of cardiac sympathetic/parasympathetic imbalance, expressed as increased LF/HF ratio.

	Odds ratio and 95% CI	***P* values**
Unadjusted model		
NAFLD and T2DM status		
Without NAFLD/Without T2DM (n=115)	*Ref*.	
With T2DM/Without NAFLD (n=56)	1.37 (0.64 – 2.94)	0.425
With NAFLD/Without T2DM (n=68)	2.63 (1.32 – 5.23)	0.006
With NAFLD/With T2DM (n=117)	3.48 (1.92 – 6.30)	<0.001
Adjusted model 1		
NAFLD and T2DM status		
Without NAFLD/Without T2DM (n=115)	*Ref*.	
With T2DM/Without NAFLD (n=56)	1.50 (0.69 – 3.29)	0.310
With NAFLD/Without T2DM (n=68)	2.69 (1.34 – 5.44)	0.006
With NAFLD/With T2DM (n=117)	3.35 (1.83 – 6.12)	<0.001
Age (years)	0.99 (0.97 – 1.01)	0.304
Sex (women vs. men)	0.49 (0.31 – 0.80)	0.004
Adjusted model 2		
NAFLD and T2DM status		
Without NAFLD/Without T2DM (n=115)	*Ref*.	
With T2DM/Without NAFLD (n=56)	1.77 (0.74 – 4.23)	0.201
With NAFLD/Without T2DM (n=68)	3.41 (1.59 – 7.29)	0.002
With NAFLD/With T2DM (n=117)	4.49 (1.90 – 10.6)	<0.001
Age (years)	0.98 (0.96 – 1.01)	0.241
Sex (women vs. men)	0.48 (0.29 – 0.80)	0.004
Body mass index (kg/m^2)	0.97 (0.91 – 1.04)	0.401
Hypertension (yes vs. no)	0.94 (0.54 – 1.64)	0.834
Dyslipidemia (yes vs. no)	0.93 (0.55 – 1.57)	0.795
HbA1c (%)	0.88 (0.58 – 1.34)	0.554
HOMA-IR score	1.01 (0.92 – 1.10)	0.890
C-reactive protein (mg/dl)	1.00 (0.49 – 2.02)	0.999
Liver stiffness (kPa)	0.93 (0.83 – 1.04)	0.203

Sample size, *n*=356. Data are expressed as odds ratio(s) and 95% confidence intervals (CI) by logistic regression analysis.
Presence of cardiac sympathetic/parasympathetic imbalance was the dependent variable in all regression models, and it was defined as increased LF/HF ratio (3rd tertile vs. 2nd and 1st tertiles combined). In particular, 119 subjects were in the 3rd tertile of LF/HF ratio [mean (SD): 6.5 ± 3.1], 119 in the 2nd tertile [mean (SD): 2.5 ± 0.5] and 118 subjects in the 1st tertile [mean (SD): 1.1 ± 0.4], respectively. *Ref*, reference category.

FIGURE LEGEND

Figure 1. Box plots of the continuous LF/HF ratio values (panel A) and the proportion of subjects with a high LF/HF ratio (panel B), i.e., defined as those belonging to the upper tertile of distribution of the LF/HF ratio, in individuals stratified in four groups according to NAFLD and T2DM status. For panel A, the central rectangle spans the 1st quartile to the 3rd quartile (i.e., the interquartile range [IQR]). The segment inside the rectangle shows the median value and the ''whiskers'' above and below the box show the locations of 1.5 x IQR values. Differences among the four groups of individuals were tested either by the Kruskal-Wallis test (panel A) or by the chi-squared test (panel B). In panel A, the inter-group differences were tested by the Dunn's post-hoc test.

REFERENCES

[1] Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology. 2016;64:73-84.

[2] Younossi 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;71:793-801.

[3] Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. Hepatology. 2018;67:328-57.

[4] European Association for the Study of the L, European Association for the Study of D, European Association for the Study of O. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. J Hepatol. 2016;64:1388-402.

[5] Byrne CD, Targher G. NAFLD: a multisystem disease. J Hepatol. 2015;62:S47-64.

[6] Anstee QM, Mantovani A, Tilg H, Targher G. Risk of cardiomyopathy and cardiac arrhythmias in patients with nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol. 2018;15:425-39.

[7] Mantovani A, Scorletti E, Mosca A, Alisi A, Byrne CD, Targher G. Complications, morbidity and mortality of nonalcoholic fatty liver disease. Metabolism. 2020;111S:154170.

[8] Byrne CD, Targher G. NAFLD as a driver of chronic kidney disease. J Hepatol. 2020;72:785-801.

[9] Targher G, Byrne CD, Tilg H. NAFLD and increased risk of cardiovascular disease: clinical associations, pathophysiological mechanisms and pharmacological implications. Gut. 2020;69:1691-705.

[10] Hadaya J, Ardell JL. Autonomic Modulation for Cardiovascular Disease. Front Physiol. 2020;11:617459.

[11] Khemani P, Mehdirad AA. Cardiovascular Disorders Mediated by Autonomic Nervous System Dysfunction. Cardiol Rev. 2020;28:65-72.

[12] Podrid PJ, Myerburg RJ. Epidemiology and stratification of risk for sudden cardiac death. Clin Cardiol. 2005;28:I3-11.

[13] Goldberger JJ, Cain ME, Hohnloser SH, Kadish AH, Knight BP, Lauer MS, et al. American Heart Association/american College of Cardiology Foundation/heart Rhythm Society scientific statement on noninvasive risk stratification techniques for identifying patients at risk for sudden cardiac death: a scientific statement from the American Heart Association Council on Clinical Cardiology Committee on Electrocardiography and Arrhythmias and Council on Epidemiology and Prevention. Heart Rhythm. 2008;5:e1-21.

[14] Thayer JF, Yamamoto SS, Brosschot JF. The relationship of autonomic imbalance, heart rate variability and cardiovascular disease risk factors. Int J Cardiol. 2010;141:122-31.

[15] Manolis AJ, Poulimenos LE, Kallistratos MS, Gavras I, Gavras H. Sympathetic overactivity in hypertension and cardiovascular disease. Curr Vasc Pharmacol. 2014;12:4-15.

[16] Boudet G, Walther G, Courteix D, Obert P, Lesourd B, Pereira B, et al. Paradoxical dissociation between heart rate and heart rate variability following different modalities of exercise in individuals with metabolic syndrome: The RESOLVE study. Eur J Prev Cardiol. 2017;24:281-96.

[17] Heart rate variability: standards of measurement, physiological interpretation and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. Circulation. 1996;93:1043-65.

[18] Liu YC, Hung CS, Wu YW, Lee YC, Lin YH, Lin C, et al. Influence of non-alcoholic fatty liver disease on autonomic changes evaluated by the time domain, frequency domain, and symbolic dynamics of heart rate variability. PLoS One. 2013;8:e61803.

[19] Kumar MS, Singh A, Jaryal AK, Ranjan P, Deepak KK, Sharma S, et al. Cardiovascular Autonomic Dysfunction in Patients of Nonalcoholic Fatty Liver Disease. Int J Hepatol. 2016;2016:5160754.
[20] Houghton D, Zalewski P, Hallsworth K, Cassidy S, Thoma C, Avery L, et al. The degree of hepatic steatosis associates with impaired cardiac and autonomic function. J Hepatol. 2019;70:1203-13.
[21] Pattaro C, Gogele M, Mascalzoni D, Melotti R, Schwienbacher C, De Grandi A, et al. The Cooperative Health Research in South Tyrol (CHRIS) study: rationale, objectives, and preliminary results. J Transl Med. 2015;13:348.
[22] Noce D, Gogele M, Schwienbacher C, Caprioli G, De Grandi A, Foco L, et al. Sequential recruitment of study participants may inflate genetic heritability estimates. Hum Genet. 2017;136:743-57.
[23] American Diabetes A. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2021. Diabetes Care. 2021;44:S15-S33.
[24] Motta BM, Grander C, Gogele M, Foco L, Vukovic V, Melotti R, et al. Microbiota, type 2 diabetes and non-alcoholic fatty liver disease: protocol of an observational study. J Transl Med. 2019;17:408.
[25] Nishida C, Ko GT, Kumanyika S. Body fat distribution and noncommunicable diseases in populations: overview of the 2008 WHO Expert Consultation on Waist Circumference and Waist-Hip Ratio. Eur J Clin Nutr. 2010;64:2-5.
[26] Garcia-Larsen V, Luczynska M, Kowalski ML, Voutilainen H, Ahlstrom M, Haahtela T, et al. Use of a common food frequency questionnaire (FFQ) to assess dietary patterns and their relation to allergy and asthma in Europe: pilot study of the GA2LEN FFQ. Eur J Clin Nutr. 2011;65:750-6.
[27] Chinn S, Jarvis D, Melotti R, Luczynska C, Ackermann-Liebrich U, Anto JM, et al. Smoking cessation, lung function, and weight gain: a follow-up study. Lancet. 2005;365:1629-35; discussion 00-1.
[28] Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, 3rd, Feldman HI, et al. A new equation to estimate glomerular filtration rate. Ann Intern Med. 2009;150:604-12.
[29] Castera L, Vilgrain V, Angulo P. Noninvasive evaluation of NAFLD. Nat Rev Gastroenterol Hepatol. 2013;10:666-75.
[30] Ballestri S, Lonardo A, Romagnoli D, Carulli L, Losi L, Day CP, et al. Ultrasonographic fatty liver indicator, a novel score which rules out NASH and is correlated with metabolic parameters in NAFLD. Liver Int. 2012;32:1242-52.
[31] Castera L. Assessing liver fibrosis. Expert Rev Gastroenterol Hepatol. 2008;2:541-52.
[32] Vuppalanchi R, Siddiqui MS, Van Natta ML, Hallinan E, Brandman D, Kowdley K, et al. Performance characteristics of vibration-controlled transient elastography for evaluation of nonalcoholic fatty liver disease. Hepatology. 2018;67:134-44.
[33] 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;51:454-62.
[34] Siddiqui MS, Vuppalanchi R, Van Natta ML, Hallinan E, Kowdley KV, Abdelmalek M, et al. Vibration-Controlled Transient Elastography to Assess Fibrosis and Steatosis in Patients With Nonalcoholic Fatty Liver Disease. Clin Gastroenterol Hepatol. 2019;17:156-63 e2.
[35] Murgia F, Melotti R, Foco L, Gogele M, Meraviglia V, Motta B, et al. Effects of smoking status, history and intensity on heart rate variability in the general population: The CHRIS study. PLoS One. 2019;14:e0215053.
[36] Perini R, Veicsteinas A. Heart rate variability and autonomic activity at rest and during exercise in various physiological conditions. Eur J Appl Physiol. 2003;90:317-25.

[37] Clough GF, Chipperfield AJ, Thanaj M, Scorletti E, Calder PC, Byrne CD. Dysregulated Neurovascular Control Underlies Declining Microvascular Functionality in People With Non-alcoholic Fatty Liver Disease (NAFLD) at Risk of Liver Fibrosis. Front Physiol. 2020;11:551.
[38] Ozveren O, Dogdu O, Sengul C, Cinar V, Eroglu E, Kucukdurmaz Z, et al. Deterioration of heart rate recovery index in patients with non-alcoholic fatty liver disease (NAFLD). Med Sci Monit. 2014;20:1539-43.
[39] Cho KI, Jo EA, Cho SH, Kim BH. The Influence of Epicardial Fat and Nonalcoholic Fatty Liver Disease on Heart Rate Recovery in Metabolic Syndrome. Metab Syndr Relat Disord. 2017;15:226-32.
[40] Liao D, Cai J, Brancati FL, Folsom A, Barnes RW, Tyroler HA, et al. Association of vagal tone with serum insulin, glucose, and diabetes mellitus--The ARIC Study. Diabetes Res Clin Pract. 1995;30:211-21.
[41] Yazdanpanah MH, Sayyadipoor S, Hojati SR, Nikmanesh A, Farjam M, Homayounfar R. The Association of Metabolic Syndrome and Its Components with Electrocardiogram Parameters and Abnormalities Among an Iranian Rural Population: The Fasa PERSIAN Cohort Study. Diabetes Metab Syndr Obes. 2020;13:2975-87.
[42] Zoppini G, Cacciatori V, Raimondo D, Gemma M, Trombetta M, Dauriz M, et al. Prevalence of Cardiovascular Autonomic Neuropathy in a Cohort of Patients With Newly Diagnosed Type 2 Diabetes: The Verona Newly Diagnosed Type 2 Diabetes Study (VNDS). Diabetes Care. 2015;38:1487-93.
[43] Mantovani A, Rigolon R, Mingolla L, Pichiri I, Cavalieri V, Salvotelli L, et al. Nonalcoholic fatty liver disease is associated with an increased prevalence of distal symmetric polyneuropathy in adult patients with type 1 diabetes. J Diabetes Complications. 2017;31:1021-6.
[44] Vincent AM, Callaghan BC, Smith AL, Feldman EL. Diabetic neuropathy: cellular mechanisms as therapeutic targets. Nat Rev Neurol. 2011;7:573-83.