Research article

JHEP|Reports

(^{13}C)-Methacetin breath test provides evidence of subclinical liver dysfunction linked to fat storage but not lifestyle

Emilio Molina-Molina,[1] Harshitha Shanmugam,[1] Agostino Di Ciaula,[1] Ignazio Grattagliano,[2] Domenica Maria Di Palo,[1] Vincenzo O. Palmieri,[1] Piero Portincasa[1,*]

[1]*Clinica Medica 'A. Murri', Department of Biomedical Sciences and Human Oncology, University of Bari Aldo Moro, Bari, Italy;* [2]*Italian College of Family Medicine, Bari, Italy*

JHEP Reports **2021.** https://doi.org/10.1016/j.jhepr.2020.100203

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is characterised by the presence of hepatic steatosis in the absence of other causes of secondary hepatic fat accumulation, and is usually associated with visceral, metabolically active obesity. However, the subclinical effects of body and liver fat accumulation on liver function are still unclear.
Methods: We used orally administered (^{13}C)-methacetin and breath test to quantify the efficiency of hepatic extraction from portal blood flow and liver microsomal function in 81 participants, in relation to presence/absence of ultrasonographic NAFLD, extent of body fat accumulation, insulin resistance, dietary models, and lifestyle.
Results: NAFLD was present in 23% of participants with normal weight, and prevalence increased with body fat and insulin resistance. Fat accumulation, NAFLD, and insulin resistance were associated with decreased hepatic extraction efficiency, and liver microsomal function was impaired in moderate-to-severe NAFLD. Caloric intake, dietary models, and lifestyles had a minor role in promoting functional changes.
Conclusions: The interplay between body fat accumulation, insulin resistance, and NAFLD is linked with altered hepatic extraction efficiency from blood flow and deranged microsomal function. Non-invasive diagnosis of subclinical alterations of liver function is relevant for primary and secondary prevention measures. Furthermore, the occurrence of NAFLD in lean individuals and the evidence that caloric intake, dietary models, and lifestyle played a minor role require further studies exploring the role of environmental factors in the natural history of these diseases.

Introduction

Obesity has reached worldwide epidemic proportions and is often associated with non-alcoholic fatty liver disease (NAFLD) (*i.e.* presence of hepatic steatosis in the absence of significant alcohol consumption or other known causes of secondary hepatic fat accumulation). NAFLD has a worldwide prevalence of almost 30% and has become the most frequent cause of chronic liver disease.

Obesity, diabetes, dyslipidaemia, hypertension, sedentary lifestyles, and metabolic syndrome are well-known risk factors for NAFLD.[1,2] Based on these risk factors, a group of experts recently proposed to introduce the term 'metabolically associated fatty liver disease'.[3] NAFLD can also develop in lean (likely metabolically impaired) individuals.[1] NAFLD develops with over-accumulation (>5% hepatocytes) of (mainly) triglycerides in hepatocytes, leading to a spectrum of conditions ranging from simple non-alcoholic fatty liver (NAFL) without significant inflammation to non-alcoholic steatohepatitis (NASH) affecting about 5% of the population, when hepatic steatosis parallels pericellular fibrosis, ballooning degeneration of hepatocytes, and lobular inflammation.[4] NASH, in turn, may progress to cirrhosis in 20% of cases. Hepatocellular carcinoma can originate from cirrhosis, but also from non-cirrhotic NAFLD.[5] NAFLD prevalence and liver fibrosis increase with age.[6]

The pathophysiology of NAFLD is complex, multifactorial, and partially unknown.[1] One aspect is that initial fat storage in the liver could drive subclinical liver abnormalities.

In particular, NAFLD development and progression increase the risk for advanced liver disease and liver-related mortality,[6] as well as non-liver-related complications, such as cardiovascular disease and malignancy.[7] In addition, patients with NAFLD suffer from poor quality of life compared with healthy individuals, mostly in the physical health component. Greater deficits occur with more advanced liver diseases, such as liver cirrhosis.[8]

Thus, a comprehensive understanding of the relationships between body fat accumulation, increased liver fat storage, and subclinical alterations of liver function might be of great interest in terms of both primary and secondary prevention of hepatic and systemic diseases, and for designing novel and efficient therapeutic interventions.

The diagnosis of NAFLD relies on both non-invasive and invasive, morphological, functional, and often complementary

Keywords: Body mass index; Liver function; Microsomal function; Non-alcoholic fatty liver disease; Portal blood flow.
Received 11 August 2020; received in revised form 24 September 2020; accepted 18 October 2020; available online 4 November 2020
* Corresponding author. Address: Clinica Medica 'Augusto Murri', Department of Biomedical Sciences and Human Oncology, University of Bari Medical School, Piazza Giulio Cesare 11, 70124 Bari, Italy. Tel.: +39 80 5478 227; fax: +39 80 5478 232.
E-mail address: piero.portincasa@uniba.it (P. Portincasa).

approaches. Relevant alcohol consumption, other causes of liver steatosis, and other underlying chronic liver diseases must be ruled out. Liver biopsy and histology remain the gold standard for diagnosis or to assess the degree of liver injury. However, liver biopsy is invasive; poorly accepted by patients; and not totally free from complications, which include pneumothorax, pain, or significant bleeding.[9] In addition, liver biopsy becomes unrealistic in routine clinical practice because of the large number of patients with NAFLD as well as several other limitations, mainly attributable to sampling variability,[10] intra- and inter-observer variability,[11] and a possible under- or over-estimation of the liver fibrosis. Liver biopsy is therefore reserved to the subset of participants with unclear diagnosis, or to predict the severity of liver disease.[12] Laboratory tests (*i.e.* serum aminotransferase levels and ferritin or gamma-glutamyl transferase [GGT] levels) can be either normal or abnormal, but are insufficient to establish a diagnosis of any subtype of NAFLD.[13] A surrogate marker of NAFLD, namely, the fatty liver index (FLI), correlates with intrahepatic fat content.[14] Imaging (ultrasonography for hyperechoic texture, computed tomography, and nuclear magnetic resonance) can detect steatosis as well as cirrhosis, but not the ongoing necro-inflammatory NASH.[15] The presence of advanced fibrosis is detectable by scoring measuring liver stiffness by non-invasive transient elastography or by applying specific algorithms, such as NAFLD fibrosis score (which considers a patient's age, BMI, hyperglycaemia, aminotransferase levels, platelet count, and albumin).

None of the aforementioned diagnostic tools provide information on the 'dynamic' functional reserve of the liver as the result of complex metabolic processes. Breath tests (BTs) are dynamic diagnostic tools using substrates processed at different levels in the gastrointestinal tract. Metabolisation processes produce gases (*i.e.* CO_2 and H_2) transferred to blood and promptly detectable in expired air. BTs are relatively simple, safe, and non-invasive tools that provide information in different clinical settings in relation to the chosen substrate. For the liver, BTs employ specific substrates labelled with the naturally occurring (^{13}C) stable (non-radioactive) isotope. The substrate metabolism at specific levels produces (^{13}C)O_2 detected in breath[16,17] as a marker of hepatic clearance of metabolically active substances.[18] Examples of (^{13}C)-substrates include aminopyrine, phenacetin, caffeine, lidocaine, methacetin and erythromycin (targeting hepatocyte microsomal function), phenylalanine and galactose (targeting cytosolic enzymatic activity), methionine, ketoisocaproic acid (KICA), and octanoic acid (targeting mitochondrial function). (^{13}C)-Methacetin is rapidly metabolised, has a low cost, lacks toxicity,[19] and is available as (^{13}C)-methacetin breath test (MBT) for investigation of liver microsomal function in chronic liver diseases,[20] including chronic HCV infection[21] and liver cirrhosis.[22] (^{13}C)-MBT could separate patients with normal/NAFL from patients with NASH,[23,24] and was predictive for F3 or F4 fibrosis score in patients with histologically proven NASH.

Because of the complex factors involved in NAFLD pathogenesis, we aimed to study liver function non-invasively by (^{13}C)-MBT according to body weight and ultrasonographic NAFLD.

In addition, we explored the interplay between several anthropometric variables, liver ultrasonography, lifestyle, and psychological components to unravel the effects of body and liver fat storage on subclinical hepatic dysfunction.

Patients and methods

Participants

In total, 81 Caucasians participated in the study (males:females = 45:36; age 45.5 ± SE 1.9 and 41.9 ± SE 2.7 years, respectively) (Table S1). The recruitment period was concluded in 6 months. The participants were consecutively enrolled at the outpatient clinic, with a previous diagnosis of metabolic disorders and/or liver steatosis, as previously assessed by a routine ultrasonography (US). Controls were academic or hospital employees at the division of internal medicine of a large regional hospital ("Policlinico") in Bari, Italy. The participants were classified according to BMI and liver steatosis at ultrasonography. We excluded main and most frequent causes of different chronic liver disease (viral, alcoholic, drug-induced damage, and autoimmune diseases) after history, physical examination, and blood samples for the determination of hepatitis B/C viral markers and autoantibodies.

The protocol included anthropometric evaluation, completion of specific questionnaires, assessment of liver function by (^{13}C)-MBT, ultrasonographic measurement of liver steatosis and visceral fat thickness, elastography assessment of the degree of liver fibrosis, calculation of the FLI, and essential blood analyses. All participants underwent the first screening visit at the outpatient clinic and the complete assessment required for about 4 h.

Anthropometric measurements

We measured body weight (kg), height (m), and calculated BMI (*i.e.* Quetelet's index as kilograms divided by metre squared [kg/m^2]). BMI ranging from 18.5 to 24.9 kg/m^2 defined participants with normal weight. BMI ranging from 25.0 to 29.9 kg/m^2 defined participants who were overweight, while BMI ≥30 kg/m^2 defined participants with obesity. Waist circumference was a marker of abdominal (visceral) fat, measured using a non-stretching tape at the superior border of the iliac crest according to the indications of the Revised National Cholesterol Education Programme-Adult Treatment Panel III (R-ATPIII), and between the iliac crest and the lower border of ribs according to the indications of the International Diabetes Federation (IDF). According to R-ATPIII, cut-off values are 88 and 102 cm for females and males, respectively.[25] For IDF, cut-off values are 80 and 94 cm for Caucasian females and males, respectively.[26]

Questionnaires

We assessed the level of physical activity, adherence to Mediterranean diet (MD), a 'junk' score, energy intake, health-related quality of life (HRQoL), and major depression by specific questionnaires.

The validated physical activity-designed questionnaire (International Physical Activity Questionnaire; long-format version) provides the simple and rapid evaluation of physical activity during leisure, occupational, and sedentary time on a weekly basis.[27] Physical activity levels were calculated based on metabolic equivalent tasks (METs), with 1 MET equal to 3.5 ml of O_2/kg/min. MET thresholds constitute valid indicators of physical activity intensity with relatively high accuracy.[28]

The MD adherence score relies on an 18-point scale (0 point = lowest adherence to 18 points = highest adherence), using a validated questionnaire.[29]

The junk score was calculated based on daily consumption of 7 high-fat high-sugar food items (ice cream, milk chocolate, chips, soft drinks, confectioned juices, French fries, as well as

typical Italian 'fast food') after completion of a validated questionnaire assessing food frequency.[30] Energy intake (kcal/day) was also calculated based on daily consumption of each macronutrient, from the aforementioned questionnaire. The HRQoL was measured by a validated 36-Item Short Form Health Survey Questionnaire (SF-36),[31] with per cent scores provided across 8 domains, that is:

- limitations in physical activities because of health problems;
- limitations in social activities because of physical or emotional problems;
- limitations in usual role activities because of physical health problems;
- bodily pain;
- general mental health (psychological distress and well-being);
- limitations in usual role activities because of emotional problems;
- vitality (energy and fatigue); and
- general health perceptions.

Scores from the short-form 6 dimension questionnaire (SF-6D) were calculated and summarised as per cent of scores 1, 3, 4, and 7 for physical health and scores 2, 5, 6, and 8 for mental health components.

For evaluation of major depression, we used the Patient Health Questionnaire-9, a previously validated questionnaire with a score ranging from 0 to 27. Major depression is diagnosed if 5 or more of the 9 depressive symptom criteria have been present at least 'more than half the days' in the past 2 weeks, and 1 of the symptoms is depressed mood or anhedonia. Other depression is diagnosed if 2, 3, or 4 depressive symptoms have been present at least more than half the days in the past 2 weeks, and 1 of the symptoms is depressed mood or anhedonia. One of the 9 symptom criteria ('thoughts that you would be better off dead or of hurting yourself in some way') counts if present at all, regardless of duration.[32]

Measurement of liver function by (^{13}C)-MBT

The protocol of (^{13}C)-MBT is described elsewhere in detail[23,33–36] (Fig. S1). In brief, the test relies on the capacity of hepatic cytochrome P450 1A2 to demethylate the ingested dose of (^{13}C)-labelled methacetin into acetaminophen and (^{13}C)-formaldehyde. After, (^{13}C)O_2 is produced and detectable in expired breath[37] (Fig. S2). The test is performed in the morning with the participants fasting for at least 8 h. Half an hour before and during the whole test, the participant refrains from smoking or from vigorous physical exercise.[24,38] We recorded information about current medical prescription and drugs able to affect liver function. Samples of expired air were collected at baseline in duplicate with a straw into glass exetainers. Then, each participant ingested 75 mg of (^{13}C)-methacetin (AB-^{13}C METACETINA®; AB ANALITICA SRL, Padua, Italy) diluted in 25 ml of still water. Breath samples were collected again at 15 and 30 min. For the interpretation of results, as stated by the manufacturer, a value of delta over baseline (DOB) of <14.5‰ after 15 min (DOB_{15}) indicates limited hepatic function and extraction ability from blood flow.[33,35] A value of cumulative per cent dose recovery (cPDR) of <8.1% after 30 min ($cPDR_{30}$) reflects methacetin cumulative oxidation percentage over time,[39] thus being an expression of reduced liver microsomal function.[23,33,35,36]

Liver steatosis

We assessed liver steatosis and visceral fat thickness by ultrasonography (Noblus-E; Hitachi Medical, Tokyo, Japan) using 3.5 MHz convex probes. Kidney cortex echogenicity was the control parenchyma against the echogenicity of the liver parenchyma (*i.e.* isoechoic normal liver or hyperechoic 'bright' steatotic liver). Ultrasonography reliably detects a hyperechoic texture upon diffuse fatty infiltration. This finding is a sensitive marker of liver steatosis ranging from Grade 0 (absent: normal liver) to Grade 1 (mild: minor increase in liver echogenicity) and Grade 2 (moderate to severe: marked increase in liver echogenicity, poor penetration of posterior segment from the right lobe, and poor or any visual images from the hepatic vessels and diaphragm), although its accuracy is poor for mild steatosis (<30%) and for the detection of underlying inflammation.[40] The FLI is an algorithm incorporating BMI, waist circumference, serum triglycerides, and GGT, and represents an additional marker of liver steatosis, with an accuracy of 0.84 (95% CI 0.81–0.87). In a scale of 0–100, a value of <30 (negative likelihood ratio = 0.2) rules out NAFLD, while an FLI ≥60 (positive likelihood ratio = 4.3) is representative of NAFLD.[41]

Liver fibrosis

The degree of liver fibrosis was assessed non-invasively by acoustic radiation force impulse (ARFI) imaging, using the equipment LOGIQ™ E9 (GE Healthcare) with a 3.5 MHz convex probe. The operator performs 10 measurements in each participant, focusing on the liver parenchyma, and cut-off values are calculated from the mean of measurements (<1.19 = F0, no fibrosis; 1.19–1.32 = F1, portal fibrosis without septa; 1.32–1.71 = F2, few septa; 1.71–2.0 = F3, numerous septa without cirrhosis; >2.0 = F4, cirrhosis).[42]

Blood analysis (serum liver enzymes)

Serum liver enzymes included alanine aminotransferase (ALT), aspartate aminotransferase (AST), and GGT measured in the fasting participant. Peripheral venous blood (2.5 ml) was drawn into serum-separating test tubes. Within 30 min, samples were centrifuged at 3,000 rpm for 10 min at room temperature, generating the 1-step centrifugation serum sample (about 1 ml). Enzymes were measured by using commercially available assay kits (Sigma-Aldrich SRL, Milan, Italy). Normal upper values were 40 IU/L for AST and ALT. Normal range of GGT was 10–50 IU/L. The Homeostatic Model Assessment for Insulin Resistance (HOMA index) measured the severity of insulin resistance in our cohort, as it is a sensitive and non-invasive method in assessing the severity of NAFLD. The HOMA index was calculated by the following formula: (plasma glucose [mg/dl] × plasma insulin [μU/ml])/405.[43]

Visceral and subcutaneous fat measurement

Visceral fat thickness was measured by ultrasonography (Noblus-E) with a 3.5 MHz convex probe. The electronic caliper measured the distance from the peritoneum boundary to the linea alba. Measurements were recorded and coded as Vmax to indicate the visceral fat measured at 2 locations, close to the xyphoid, and visceral fat was measured at the widest point.[44]

Subcutaneous fat thickness was measured using a 5 MHz convex probe at 2 sites. The first site was in the midline just below the xiphoid process in front of the left lobe of the liver (LSFT). The second site was just to the right of the umbilicus (USFT).[43]

Statistical analysis

Data are presented as mean ± SEM or as percentages. One-way ANOVA assessed inter-group differences. Differences between 2 groups were tested by Student's *t* test for unpaired data. The Chi-square test was used to compare proportions. Pearson correlation coefficient was used for correlations. Wilks' lambda test was used for multivariate analysis of covariance; https://www.ncss.com/software/ncss/. Graphic representation of data is provided by SigmaPlot software (https://systatsoftware.com/products/sigmaplot/).

The sample size was calculated based on results from previous studies[23] and using the α coefficient of 0.05 for a normally distributed population and set the power at 0.80 (Calculator.net; https://www.calculator.net/sample-size-calculator) for parameters of BT. To calculate odds ratios (ORs) and CIs for the ultrasonographic score of NAFLD associated with measurement of liver function by (^{13}C)-MBT, separate logistic regression models were fitted. DOB_{15} as marker of extraction efficiency from portal blood flow and $cPDR_{30}$ as a marker of liver microsomal function were the dependent variables, and the ultrasonographic score of liver steatosis was considered as the independent variable. The models were adjusted according to possible confounders. Models were fitted using R software, version 3.1.1 (The R Foundation for Statistical Computing, Vienna, Austria). The effects of morphological anthropometric and metabolic parameters (NAFLD, overweight/obesity, and liver transaminases) on microsomal function and the extent of hepatic extraction efficiency from portal blood flow, determined in all the participants included in the study (n = 81), were calculated as coefficients in a retrospective model of multiple logistic regression with participant groups (absent compared with present) as independent factors. The sensitivity of the BT and positive and negative predictive values were calculated to differentiate participants. Results were considered significant at the 5% critical level (p <0.05). Statistical analyses were performed with NCSS10 statistical software (NCSS, LLC, Kaysville, UT, USA).

Study approval

The protocol was approved by the local ethics committee (study number 5408; protocol number 0013869; AOUCPG23/COMET/P). Before the study, all participants gave full written informed consent to allow all authors to access and use the data for research purposes.

Results

Anthropometric, clinical, and metabolic features according to BMI

The participants were stratified according to BMI (*i.e.* normal weight, N = 26, 32%; overweight, N = 28, 35%; obese, N = 27, 33%) and compared for general clinical features, anthropometric and liver characteristics, and questionnaires (Table S1). Males were mostly overweight (75%) and with obesity (59%), while females were mostly of normal weight (69%). Participants with normal weight (otherwise completely healthy) were about 10 years younger than participants who were overweight and with obesity. Systolic and diastolic blood pressure increased significantly in participants who were overweight/with obesity compared with those with normal weight. Smoking habits were comparable between the 3 subgroups.

The analyses of body fat distribution confirmed that BMI, waist circumferences according to both criteria (*i.e.* R-ATPIII and IDF), and visceral fat thickness (ultrasonography) increased progressively in participants who were lean, overweight, and with obesity, irrespective of gender.

NAFLD (a 'bright liver' at ultrasonography) was present in 54/81 (67%) participants. This finding was also present in participants with normal weight (23%), although the overall mean ultrasonographic score and FLI remained very low. With increasing BMI, however, the prevalence of NAFLD progressively increased (from 23% in participants with normal weight to 79% in participants who were overweight and 96% in participants with obesity). Similar trends were also evident for NAFLD score (0.1 ± 0.1, 1.0 ± 0.1, and 1.6 ± 0.1, respectively) and FLI (10.3 ± 1.8, 50.4 ± 4.4, and 81.2 ± 3.3, respectively). In line with such findings, the ARFI fibrosis score increased mildly but significantly in participants with obesity.

Concerning the biochemical markers, serum ALT increased mildly but significantly within the normal cut-off value in participants who were overweight and with obesity. However, the rate of participants with abnormal ALT levels was significantly greater in participants who were overweight (21%) and with obesity (37%). The levels of AST and GGT did not differ across the 3 subgroups, despite the prevalence of individuals with abnormal levels tended to increase in participants who were overweight (11%) and with obesity (22%).

Insulin resistance, as assessed by the HOMA index, increased significantly with body size. Total and LDL cholesterol were similar across the 3 subgroups. However, participants who were overweight and with obesity showed lower HDL cholesterol levels compared with participants with normal weight. The participants with obesity also showed higher triglyceride levels than participants with normal weight.

The levels of physical activity were lower in participants with obesity. The adherence score to MD was similar among groups, with a trend for progressive increase in the consumption of junk food from participants with normal weight to those who were overweight and with obesity. Daily energy intake remained comparable across groups.

Physical health, as assessed by the SF-36 questionnaire, was significantly lower in participants with obesity when compared with those with normal weight. Mental health components, however, did not differ between the 3 groups, although we observed a trend towards increased major depression scores with increasing weight and steatosis.

Anthropometric, clinical, and metabolic features according to NAFLD

We further explored the anthropometric, clinical, and metabolic features of the study group according to the presence of NAFLD (Table 1).

Most of the enrolled participants had NAFLD (60.5%), and within this subgroup, they were mostly males (63%). The participants with NAFLD were older, and had increased BMI, waist and visceral fat thickness, serum triglycerides, ALT and GGT, systo-diastolic blood pressure, and HOMA index, but lower HDL cholesterol compared with participants without NAFLD. No difference was detected with respect to smoking habits, adherence to MD, levels of physical activity, daily energy intake, and psychological profiles.

(^{13}C)-MBT for the study of dynamic liver function

All participants completed the BT study without reporting side effects. Data are presented with respect to classes of BMI

Table 1. Anthropometric, clinical, metabolic, lifestyle, quality of life, and major depression features of the study group (N = 81) according to NAFLD.

	NAFLD absent	NAFLD present
N (%)	32 (39.5)	49 (60.5)*
Males	14 (44)	31 (63)
Females	18 (56)	18 (37)†
Age (years)	36.3 ± 2.6	49.9 ± 1.8*
Normal weight	23 (72)	3 (6)*
Overweight	8 (25)	20 (41)
Obesity	1 (3)	26 (53)*
Systolic blood pressure (mmHg)	114.3 ± 2.1	124.3 ± 1.8*
Diastolic blood pressure (mmHg)	71.8 ± 1.1	79.4 ± 0.9*
Smoking habits (N cigarettes/day)	3.0 ± 1.2	2.3 ± 0.8
BMI (kg/m^2)	23.5 ± 0.5	31.4 ± 0.7*
Waist circumference, males (cm)‡	91.1 ± 2.1	109.5 ± 2.2*
Waist circumference, females (cm)‡	77.8 ± 2.4†	109.4 ± 3.3*
Waist circumference, males (cm)§	87.3 ± 2.1	104.7 ± 1.7*
Waist circumference, females (cm)§	72.5 ± 2.5†	99.4 ± 2.4*
Subcutaneous fat by US (mm)	11.3 ± 0.9	16.2 ± 0.9*
Visceral fat thickness by US (mm)	28.4 ± 2.2	59.6 ± 2.9*
NAFLD by US	32 (0)	49 (100)*
NAFLD by US, score	0	1.5 ± 0.1*
FLI	16.7 ± 3.6	67.0 ± 3.7*
Liver fibrosis score by ARFI	0.3 ± 0.1	0.9 ± 0.1*
Serum ALT (IU/L)	19.9 ± 2.0	33.0 ± 2.5*
ALT, N (%) abnormal	3 (9)	13 (27)
Serum AST (IU/L)	19.6 ± 1.5	22.7 ± 1.2
AST, N (%) abnormal	1 (3)	3 (6)
Serum GGT (IU/L)	23.2 ± 3.4	45.1 ± 7.9*
GGT, N (%) abnormal	1 (3)	9 (18)
HOMA index	1.38 ± 0.2	3.8 ± 0.3*
Total cholesterol (mg/dl)	185 ± 6	188 ± 6
HDL cholesterol (mg/dl)	61 ± 2	50 ± 2*
LDL cholesterol (mg/dl)	106 ± 5	109 ± 6
Triglycerides (mg/dl)	90 ± 8	124 ± 12*
Physical activity (METs/week)	3,520.5 ± 510.8	2,584.5 ± 445.5
Mediterranean diet adherence (score)	10.3 ± 0.4	10.3 ± 0.3
'Junk' score	96.2 ± 23.9	127.5 ± 30.6
Energy intake (kcal/day)	2,086 ± 136	2,068 ± 110
SF-36, physical health component (%)	74.8 ± 2.4	67.2 ± 2.8
SF-36, mental health component (%)	72.1 ± 3.3	68.2 ± 2.7
Major depression score	3.9 ± 0.8	5.5 ± 0.6

Data are expressed as n (%) or mean ± SEM. Significance levels: *vs. NAFLD absent; †vs. males; 0.0001< p <0.04 (Student's t test or Chi-square test, as appropriate). ‡According to the R-ATPIII. §According to the International Diabetes Federation.
ALT, alanine aminotransferase; ARFI, acoustic radiation force impulse; AST, aspartate aminotransferase; FLI, fatty liver index; GGT, gamma-glutamyltransferase; HOMA, Homeostatic Model Assessment for Insulin Resistance; MBT, methacetin breath test; MET, metabolic equivalent task; NAFLD, non-alcoholic fatty liver disease; R-ATPIII, Revised National Cholesterol Education Programme-Adult Treatment Panel III; SF-36, 36-Item Short Form Health Survey Questionnaire; US, ultrasonography.

Table 2. Results of dynamic liver function by (^{13}C)-methacetin breath test (N = 81), according to BMI.

	Normal weight (BMI: 18.5–24.9 kg/m^2)	Overweight (BMI: 25.0–29.9 kg/m^2)	Obesity (BMI ≥30.0 kg/m^2)
N (%)	26 (32)	28 (35)	27 (33)
Hepatic extraction from portal blood flow (DOB_{15})	19.1 ± 1.6	15.4 ± 1.3	12.7 ± 1.1*
Abnormal	6 (23)	15 (54)*	17 (63)*
Liver microsomal function ($cPDR_{30}$)	12.9 ± 0.8	11.7 ± 0.7	11.3 ± 0.6
Abnormal	1 (4)	2 (7)	4 (15)

Data are expressed as n (%) or mean ± SEM. Significance levels: *vs. normal weight; 0.0001< p <0.04 (Chi-square test or ANOVA followed by Fisher's LSD multiple comparison test, as appropriate). DOB_{15} is representative of hepatic extraction efficiency from portal blood flow; abnormal if <14.5‰. $cPDR_{30}$ is representative of liver microsomal function; abnormal if <8.1%.
$cPDR_{30}$, cumulative per cent dose recovery after 30 min; DOB_{15}, delta over baseline after 15 min.

Table 3. Results of dynamic liver function by (^{13}C)-methacetin breath test (N = 81), according to NAFLD.

	NAFLD absent	NAFLD present
N (%)	32 (39.5)	49 (60.5)
Hepatic extraction from portal blood flow (DOB_{15})	19.2 ± 1.4	13.4 ± 0.9*
Abnormal	8 (25)	30 (61)*
Liver microsomal function ($cPDR_{30}$)	13.0 ± 0.7	11.3 ± 0.5*
Abnormal	1 (3)	6 (12)

Data are expressed as n (%) or mean ± SEM. Significance levels: *vs. NAFLD absent; 0.0001< p <0.04 (Chi-square test or Student's t test for unpaired data, as approproate). DOB_{15} is representative of hepatic extraction efficiency from portal blood flow; abnormal if <14.5‰. $cPDR_{30}$ is representative of liver microsomal function; abnormal if <8.1%.
$cPDR_{30}$, cumulative per cent dose recovery after 30 min; DOB_{15}, delta over baseline after 15 min; NAFLD, non-alcoholic fatty liver disease.

(Table 2) and presence of NAFLD (Table 3). Concerning body size, the analysis of (^{13}C)-MBT revealed that DOB_{15}, a marker of hepatic extraction efficiency from portal blood flow, tended to decrease in participants who were overweight and decreased significantly in participants with obesity compared with those with normal weight (Fig. 1A). The rate of participants with an abnormal DOB_{15} was higher in both participants who were overweight (54%) and with obesity (63%) than in participants with normal weight (23%) (Fig. 1C). Values of $cPDR_{30}$ were similar among the 3 subgroups (Fig. 1B). The percentage of individuals with an abnormal $cPDR_{30}$, however, tended to increase with body weight (Fig. 1D).

When the participants were stratified according to the presence of NAFLD (Table 3), both DOB_{15} and $cPDR_{30}$ were significantly lower in participants with compared with those without NAFLD. Accordingly, the prevalence of individuals with abnormal DOB_{15} and $cPDR_{30}$ values was higher when NAFLD occurred.

In the whole population, we used logistic regression models to calculate the ORs relating the spectrum of NAFLD at US (US score), according to DOB_{15} and $cPDR_{30}$. The OR for DOB_{15} decrease changed with the degree of NAFLD, and was higher in the case of both mild (OR 0.91 [95% CI 0.84–0.99]) and moderate-to-severe steatosis (OR 0.84 [95% CI 0.76–0.94]) than in the reference group (normal liver at US). Results persisted after adjusting for BMI and age, considered as covariates (mild steatosis: OR 0.92 [95% CI 0.9–0.93]; moderate-to-severe steatosis: OR 0.80 [95% CI 0.78–0.82]; Fig. 2A).

The OR for a $cPDR_{30}$ decrease according to an increased US score of NAFLD was higher in the case of moderate-to-severe steatosis (OR 0.80 [95% CI 0.67–0.96]), but not in mild steatosis, than in the reference group (normal liver at US). Results persisted after adjusting for BMI and age, considered as covariates (mild steatosis: OR 0.98 [95% CI 0.95–1.01]; moderate-to-severe steatosis: OR 0.72 [95% CI 0.7–0.75]; Fig. 2C).

The average DOB_{15} was lower (p = 0.0007 ANOVA) in both subgroups of participants with mild (14.7 ± 1.4) and moderate-to-severe steatosis (12.0 ± 1.4) compared with those with normal liver at US (19.2 ± 1.2) (Fig. 2B). $CPDR_{30}$ was significantly lower in moderate-to-severe NAFLD (10.6 ± 0.7), but not in mild NAFLD (12.0 ± 0.7), compared with normal liver at US (13.0 ± 0.6, ANOVA p <0.05 followed by Fisher's least significant difference (LSD) multiple comparison test, −0.39 to 0.23; Fig. 2D).

We further explored DOB_{15} results according to the cut-off values for normal values (Fig. 3). Participants with abnormal DOB_{15} (*i.e.* <14.5‰) had a significantly increased BMI (30.2 ± 0.9

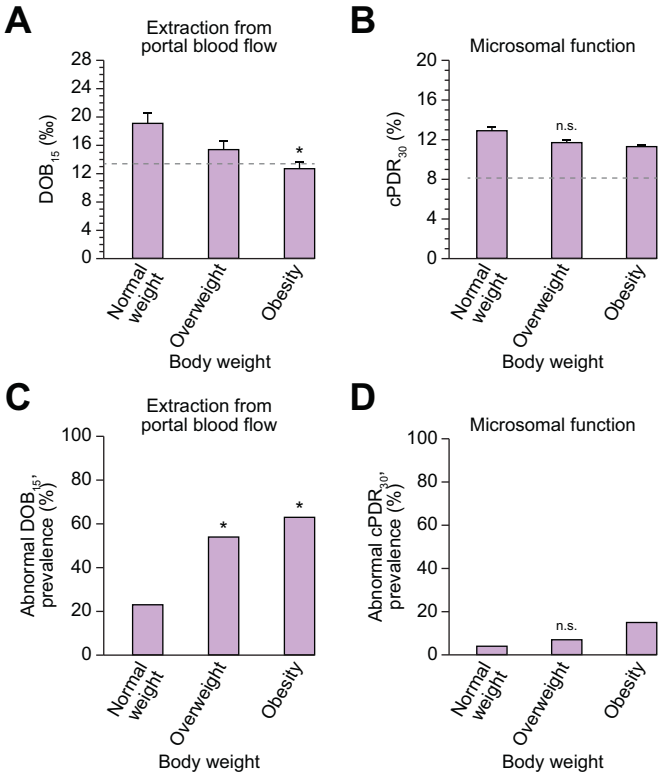

Fig. 1. Results from the breath test analysis after orally administered (^{13}C)-methacetin in participants with different BMI. Results of (^{13}C)-MBT according to body weight, as marker of hepatic extraction efficiency from (A) portal blood flow and (B) liver microsomal function. Prevalence of abnormality is depicted for hepatic extraction from (C) portal blood flow and (D) liver microsomal function. Bars represent means; vertical lines are SEM. Intermittent horizontal lines represent normal cut-off values (abnormal DOB_{15} <14.5‰; abnormal $cPDR_{30}$ <8.1%). Significance levels: *vs.* normal weight (0.0001< p <0.04, ANOVA followed by Fisher's LSD multiple comparison test). $cPDR_{30}$, cumulative per cent dose recovery after 30 min; DOB_{15}, delta over baseline after 15 min; n.s., not significant.

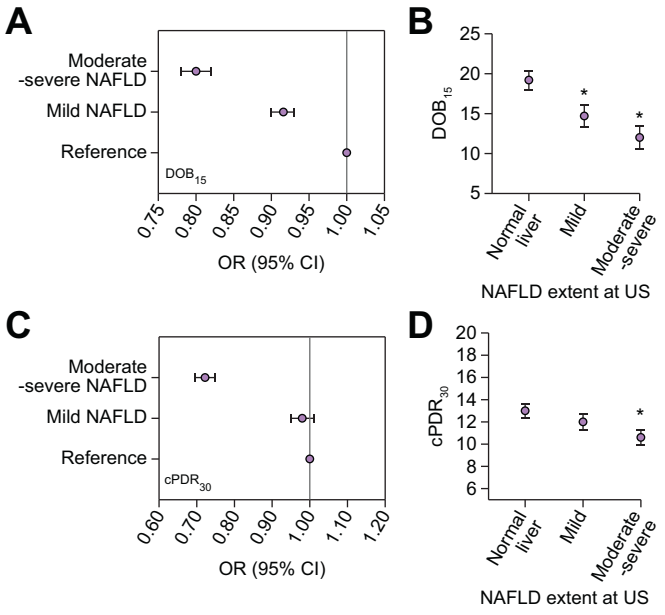

Fig. 2. ORs and analysis of variance relating the spectrum of NAFLD at ultrasonography (US score) with DOB_{15} and $cPDR_{30}$. (A) and (C) show ORs and 95% CIs relating the spectrum of NAFLD at ultrasonography (US score) with, respectively, DOB_{15} and $cPDR_{30}$. Values were calculated by logistic regression models, with DOB_{15} and $cPDR_{30}$ as dependent variables and the ultrasonographic score of NAFLD as the independent variable. Models were adjusted according to age and BMI as covariates. (B) and (D) indicate average DOB_{15} and $cPDR_{30}$, respectively, in participants grouped according to the extent of NAFLD, as assessed by ultrasonography. Data are expressed as mean ± SE. *p <0.01 *vs.* participants with normal liver at ultrasonography (ANOVA followed by Fisher's least significant difference multiple comparison test). CI, 95% confidence intervals; $cPDR_{30}$, cumulative per cent dose recovery after 30 min; DOB_{15}, delta over baseline after 15 min; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio.

vs. 26.5 ± 0.8 kg/m^2; p = 0.003), waist circumference (R-ATPIII: 103.6 ± 2.5 *vs.* 95.4 ± 2.9 cm, p = 0.016; IDF: 99.7 ± 2.3 *vs.* 87.7 ± 2.3 cm, p = 0.001), visceral fat thickness (55.5 ± 3.5 *vs.* 40.2 ± 3.5 mm; p = 0.002), ultrasonographic NAFLD score (1.2 ± 0.1 *vs.* 0.6 ± 0.1; p = 0.0000), FLI (59.6 ± 5.1 *vs.* 36.5 ± 5.3; p = 0.002), serum ALT (33.7 ± 3.0 *vs.* 22.2 ± 1.8 IU/L; p = 0.004), and serum GGT (39.7 ± 7.9 *vs.* 34.0 ± 6.9 IU/L; p = 0.048). No difference existed for $cPDR_{30}$ according to normal cut-off values (data not shown).

There was a positive and strong correlation between DOB_{15} and $cPDR_{30}$ (r = 0.887; p = 0.0000). In addition, DOB_{15} correlated negatively with BMI (r = −0.295; p = 0.008), waist circumference (IDF; r = −0.361; p = 0.001), liver steatosis (r = −0.397; p = 0.0000), visceral fat thickness (r = −0.297; p = 0.007), HOMA index (r = −0.28; p = 0.01), serum ALT (r = −0.276; p = 0.014), and FLI (r = −0.347; p = 0.002). DOB_{15} correlated positively with HDL cholesterol (r = 0.24; p = 0.03), and liver microsomal function ($cPDR_{30}$) correlated negatively with the degree of liver steatosis (r = −0.274; p = 0.013) (Table 4).

When analysing all potential confounding factors (NAFLD, age, sex, BMI, waist circumference [IDF], degree of liver steatosis and fibrosis, smoking habits, physical activity and adherence to MD), multivariate ANOVA revealed that DOB_{15} was significantly related to NAFLD and smoking habits (p = 0.019 and p = 0.041, respectively). Liver microsomal function ($cPDR_{30}$) related to NAFLD by Wilks' lambda test (p = 0.036) (Table 5).

Discussion

In this comprehensive study, we explored 2 markers of subclinical liver dysfunction (the efficiency of hepatic extraction from portal blood flow and liver microsomal functionality) by (^{13}C)-MBT, in relation to the presence of liver steatosis in participants with normal weight, who were overweight, and with obesity. Other variables involved in the analysis were anthropometric, clinical, metabolic, lifestyle, quality of life, and major depression profiles.

Role of age, gender, and body size

Enrolled were individuals with normal weight, but about 10 years younger than participants who were overweight and with obesity. Also, males were mostly overweight and with obesity compared with females. This last result confirms previous observations in the same geographical area (the Apulia region).[45]

We report a general difficulty in clinical studies to enrol totally 'healthy' individuals with normal weight, not metabolically compromised with increasing age. This finding might reflect the worrisome age-related raising prevalence rate of

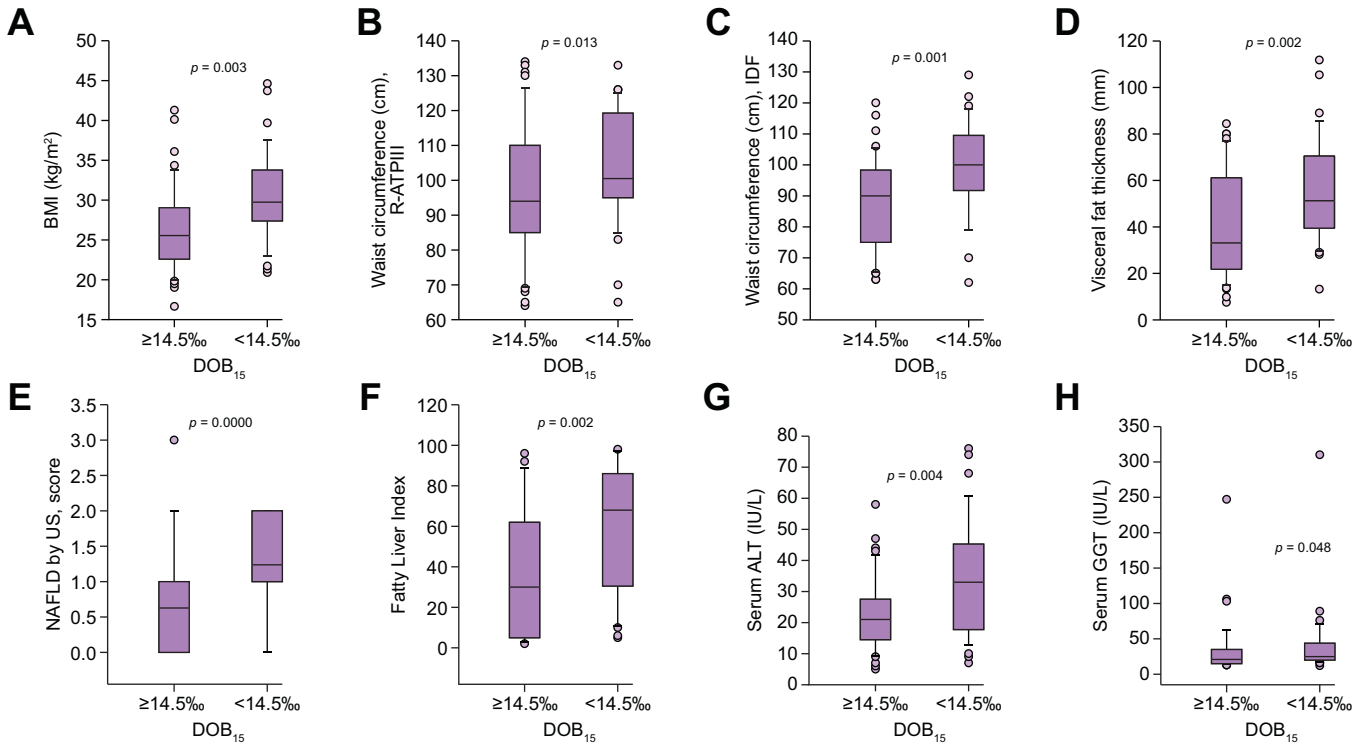

Fig. 3. DOB_{15} results according to the cut-off for normal values. Box and whiskers plots according to cut-off values of DOB_{15}. Boxes report 25th and 75th percentiles with medians at the centre. Whiskers are calculated from the IQRs. Outliers appear as dots outside the whiskers. Normal DOB_{15} ≥14.5‰; abnormal <14.5‰. Panels show changes of (A) BMI, (B) waist circumference by R-ATPIII and (C) IDF, (D) visceral fat thickness, (E) NAFLD score, (F) fatty liver index, (G) serum ALT, and (H) serum GGT. Differences were tested by Student's *t* test for unpaired data. ALT, alanine aminotransferase; DOB_{15}, delta over baseline after 15 min; GGT, gamma-glutamyl transferase; IDF, International Diabetes Federation; NAFLD, non-alcoholic fatty liver disease; R-ATPIII, Revised National Cholesterol Education Programme-Adult Treatment Panel III; US, ultrasonography.

Table 4. Linear correlations between dynamic liver function and study variables.

Dynamic liver function	Study variables	Correlation	*p* value
Hepatic extraction from portal blood flow (DOB_{15})	Microsomal function ($cPDR_{30}$)	0.887	0.0000
	Liver steatosis	−0.397	0.0000
	Waist circumference*	−0.361	0.001
	FLI	−0.347	0.002
	Visceral fat thickness	−0.297	0.007
	BMI	−0.295	0.008
	HOMA index	−0.28	0.01
	Serum ALT	−0.276	0.014
Microsomal function ($cPDR_{30}$)	Hepatic extraction from portal blood flow (DOB_{15})	0.887	0.0000
	NAFLD by US	−0.274	0.013

ALT, alanine aminotransferase; $cPDR_{30}$, cumulative per cent dose recovery after 30 min; DOB_{15}, delta over baseline after 15 min; FLI, fatty liver index; HOMA, Homeostatic Model Assessment for Insulin Resistance; NAFLD, non-alcoholic fatty liver disease; US, ultrasonography.
* According to the International Diabetes Federation.

Table 5. Multivariate ANOVA.

Dynamic liver function	Variable of study	*p* value (Wilks' lambda)
Hepatic extraction from portal blood flow (DOB_{15})	NAFLD by US	0.019
	Smoking habits (N cigarettes/day)	0.041
Microsomal function ($cPDR_{30}$)	NAFLD by US	0.036

$cPDR_{30}$, cumulative per cent dose recovery after 30 min; DOB_{15}, delta over baseline after 15 min; NAFLD, non-alcoholic fatty liver disease; US, ultrasonography.

factors contributing to the metabolic syndrome or isolated components of the metabolic syndrome.[46] However, we checked if (^{13}C)-MBT changed in relation to age, and found no significant correlation (DOB_{15}, r = 0.02, p = 0.9; $cPDR_{30}$, r = 0.01, p = 0.9). Furthermore, age as a covariate did not influence the results in logistic regression models fitted to calculate ORs for the ultrasonographic score of NAFLD, associated with measurement of liver function by (^{13}C)-MBT. Similarly, (^{13}C)-MBT was not different according to gender. In addition, we found that all metabolic variables deteriorated with increasing body size, together with the prevalence and degree of liver steatosis (79% and 96% in participants who were overweight and with obesity, respectively) and serum enzymes. Notably, slightly more than 20% of participants with normal weight had liver steatosis, and this is a major concern in current research because of potential consequences of chronic liver disease in lean individuals with 'metabolic obesity' as well as individuals without obesity.[1,47]

In the present study, however, the diagnosis of NAFLD in lean participants can be underestimated as a consequence of the poor accuracy of liver US in diagnosing the presence of a mild steatosis (*i.e.* <30%).[40]

A novel finding is that with increasing body weight and liver steatosis, the dynamic indices of liver function

deteriorate. The derangement concerned parameters related to hepatic extraction efficiency from portal blood flow and liver microsomal function, and changes were independent of gender and age.

Use of methacetin and BT for the dynamic study of the liver

Methacetin is on the market, and its use is approved in Europe and accepted by local hospital ethical boards because of its non-invasiveness and lack of potential side effects. The liver O-demethylation of (^{13}C)-methacetin occurs at the microsomal level by the cytochrome P450 1A2 (CYP1A2) with production of acetaminophen + (^{13}C)-formaldehyde and then (^{13}C) O_2, which appear in exhaled air (Fig. 2). The metabolic pathway of (^{13}C)-methacetin in the liver is summarised as DOB_{15} and $cPDR_{30}$, which become markers of liver functional 'reserve' of extraction efficiency from portal blood flow and microsomal functionality, respectively.[17,48] In a previous study, (^{13}C)-MBT predicted the risk of liver-related death and development/exacerbation of ascites more accurately than the model for end-stage liver disease score in participants with liver cirrhosis evaluated for liver transplantation.[22] (^{13}C)-MBT was safe and precise in discriminating between individuals with and without cirrhosis, with a sensitivity and specificity of 93.5% and 95%, respectively.[49] In participants with biopsy-proven advanced steatosis/NASH, we found that both microsomal (^{13}C)-MBT and mitochondrial (^{13}C)-ketoisocaproate functions were defective, with more evident damage in the case of participants with NASH.[23] In the present study, we extended the informative power of (^{13}C)-MBT in participants with early NAFLD, who were overweight, and with obesity, and related data with several metabolic and ultrasonographic parameters.

Although the results from the present study require confirmation in a larger group of individuals, evidence points to BT following oral administration of (^{13}C)-methacetin as a valuable, non-invasive, and low-cost tool for the assessment of the efficiency of hepatic extraction from portal blood flow and liver microsomal function.

Hepatic extraction efficiency in participants with obesity and NAFLD

Participants with obesity and NAFLD had decreased hepatic extraction efficiency from portal blood flow, and, in this analysis, the participants with obesity represented the most impaired group. DOB_{15} decreased by 34% in participants with obesity compared with those with normal weight. In line with these results, the prevalence of abnormal extraction efficiency from portal blood flow increased significantly in participants who were overweight (54%) and with obesity (63%) compared with those with normal weight. In parallel, all markers of liver steatosis increased with body weight. Moreover, waist circumference[50] and FLI,[41] both markers of steatosis and insulin resistance,[51] increased significantly in all participants who were overweight and with obesity, and correlated negatively with the extent of extraction efficiency from portal blood flow (DOB_{15}).

In the present series, the OR for a DOB_{15} decrease paralleled the degree of NAFLD, being higher in the case of mild and moderate-to-severe NAFLD than in participants with normal liver at US. A first explanation for decreased DOB_{15} in participants who were overweight and with obesity and NAFLD is that the intrinsic liver 'stiffness' will increase with fat deposition and possibly with initial fibrosis. This change might increase the intrahepatic resistance to blood flow, in the absence of apparent portal hypertension and splenomegaly, 2 conditions often recorded in advanced liver disease (*i.e.* liver cirrhosis). This hypothesis is supported by studies in animal models[52–54] and in humans,[55] showing an early increase in intrahepatic vascular resistance to portal blood flow during the development of disease. These effects on liver microcirculation seem to be mediated by fat accumulation, insulin resistance, sinusoidal endothelial dysfunction,[52,53,56] increased thromboxane and liver endothelin-1 expression,[53] parenchymal hypoxia,[54,56] and architectural derangement of sinusoidal anatomy.[53] In a study exploring portal pressure in individuals with NAFLD undergoing transjugular liver biopsy, the degree of steatosis was the unique factor independently predicting the presence of portal hypertension.[55]

The finding in our study is supported by the significant increase of liver fibrosis score by ARFI, especially in participants with obesity. Fibrosis assessment by ARFI has not been yet convincingly validated in NAFLD, and obesity may be considered a limiting factor for the accuracy of this diagnostic technique. However, a recent systematic review and meta-analysis suggests that ARFI is an acceptable diagnostic tool in staging hepatic fibrosis of non-viral origin, in particular in the case of severe fibrosis (F ≥3).[57]

Still, our participants had no evidence of advanced liver disease, including cirrhosis, as documented by history, physical, instrumental, and blood test analyses. Splenomegaly was also absent in all participants. Increased visceral adiposity and intra-abdominal pressure while gaining body weight,[58] therefore, could represent an additional factor playing a role in decreasing 'dynamic' portal blood flow, irrespective of gross liver function *per se*. Thus, physicians should look after several and mostly subclinical metabolic determinants in patients with NAFLD rather than advanced features of chronic liver diseases. We also found an abnormal DOB_{15} in 20% of participants with normal weight. This was the case in individuals with 'lean' NAFLD, as discussed previously.[1,47] This finding might point to a different origin of initial defective extraction efficiency from portal blood flow in individuals with normal BMI and liver steatosis (*e.g.* initial steatotic stiffness without fibrosis). More prospective studies urge to provide answers in this field.

Liver microsomal function in participants with obesity and NAFLD

In this study, $cPDR_{30}$, a marker of microsomal function, tended to decrease with increasing body weight and was significantly decreased in the presence of NAFLD, in particular in the case of moderate-to-severe steatosis.

We previously reported that methacetin demethylation occurred to a greater extent in individuals with Stages 0–III of biopsy-proven NASH compared with healthy individuals, likely reflecting increased metabolic activity of the cytochrome P450 system, in the absence of chronic ethanol consumption or medication.[23] Patients with NASH had decreased decarboxylation of KICA, pointing to an impaired metabolic pathway for branched-chain amino acids at the mitochondrial level. In other studies, patients with chronic non-cirrhotic viral hepatitis had lower methacetin demethylation capacity or were even comparable with healthy participants.[35]

Although liver biopsy was not performed in the present study because of ethical concern, liver fibrosis by ARFI was still moderate, ruling out steatohepatitis (NASH) with advanced fibrosis. We speculate that the simple accumulation of fat in the liver will

not greatly influence liver microsomal function compared with the extent of the hepatic extraction efficiency from portal blood flow.

Altered metabolic homeostasis

A type of metabolic damage to hepatocyte, however, was also possible in the present series. In our study, we observed increased serum ALT levels in both participants who were overweight and with obesity compared with those with normal weight. Although the increased serum aminotransferase levels are not representative of the severity of steatohepatitis, toxic metabolic effects secondary to severe adipose tissue insulin resistance and high liver triglyceride content are possible.[59] In accordance with previous studies, we found that (^{13}C)-MBT test is more reliable in predicting advanced fibrosis and cirrhosis than simple biochemical parameters (AST-to-platelet ratio; AST-to-ALT ratio).[34]

In the present series, levels of triglycerides were higher, and those of HDL cholesterol were lower in participants with obesity compared with those with normal weight. A comparable serum lipid profile was detected in participants with NAFLD compared with those without NAFLD. Independent relationship between TG/HDL cholesterol ratio,[60] total cholesterol/HDL cholesterol ratio,[61] and NAFLD exist, and both indices act as predictors of NAFLD.

Several redox and oxidant signalling pathways involving cholesterol could play a role in the pathogenesis of NAFLD, as loaded cholesterol in the hepatocyte can impair mitochondrial and lysosomal function. NAFLD progression would not be merely associated with excess caloric intake, but dependent on lipotoxicity in patients with NAFLD both with and without obesity.[62] These findings are in line with results from the present study, showing subclinical alterations involving microsomal activity and a poor role played by daily caloric intake, which was similar across subgroups.

In addition, a recent study found that cholesterol (free cholesterol and oxidised LDL) can accumulate in the portal vein wall, a step predisposing to portal venous NLRP3 inflammasome-mediated inflammation and fibrosis in NAFLD.[63] This situation might account for reduced DOB_{15}, an index of hepatic extraction from portal blood flow, as observed in our study in participants with obesity and those with NAFLD.

Our study provides further clues to the role of NAFLD in clinical medicine. NAFLD was associated with wide abnormalities of anthropometric, clinical, metabolic, quality of life, and major depression features, and both DOB_{15} and $cPDR_{30}$ decreased in participants with NAFLD. Most of these changes are the consequences of pathways involving a dysfunctional adipose tissue, insulin resistance, lipotoxicity, and glucotoxicity,[59,64] rather than merely dietary habits and lifestyle.

Role of diet and physical activity

MD has been previously associated with improvements in anthropometric measurements, liver enzymes, lipid profile, and NAFLD severity indices (including FLI), in participants with NAFLD. Dietary interventions are also indicated as a low-cost low-risk strategy to reduce the burden of liver diseases.[65] In our study, however, both the adherence to MD and the daily caloric intake seem to have a limited role, probably also explained by the high standard adherence to the MD in the particular population studied. In fact, MD scores and the average caloric intake were similar according to body weight and presence of NAFLD. Furthermore, when classifying groups by adherence to MD (score: 0–18; low adherence <10 or high adherence ≥10), all participants were, on average, above 10, and there were no significant differences (data not shown) concerning hepatic extraction efficiency from portal blood flow (DOB_{15}) and liver microsomal function ($cPDR_{30}$). The consumption of junk food did not seem to have a relevant role. Only a non-significant trend towards an increased junk score was noticed in participants who were overweight and with obesity compared with those with normal weight, and no difference in this index was evident when comparing participants with or without NAFLD.

The results of questionnaires also bring additional information regarding lifestyles, quality of life, and major depression in relation to BMI and NAFLD.

Physical exercise, especially aerobic exercise, has previously shown to decrease the intrahepatic fat content as well as ameliorated the liver enzymes profile. Further, other benefits of exercise include improved flow-mediated vascular dilation and cardiac function and increased oxygen uptake.[66] In our study, participants with obesity showed a reduced physical activity compared with those with normal weight. However, the extent of physical activity was similar in participants with or without NAFLD, and we found no relation between total volume of physical activity and liver function parameters by (^{13}C)-MBT.

Despite the poor role of diet and lifestyle, in the present series, as expected, the analysis of the HOMA index revealed an increased insulin resistance in participants who were overweight and with obesity compared with those with normal weight. Similarly, an increased HOMA index was evident in participants with NAFLD compared with those without NAFLD.

Quality of life in participants with obesity and NAFLD

The quality of life might deteriorate with increasing BMI and NAFLD, regardless of chronic illnesses. We confirm that physical health was significantly impaired in participants with obesity compared with those who were overweight and with normal weight, likely because of limited affordable daily activities.[67] Physical health, rather than mental health, tended to be lower with NAFLD. This finding is in line with previous reports.[8,67]

NAFLD has been previously associated with major depression in a representative sample of adults in the USA.[68] We noticed a trend towards deterioration of major depression score in both participants who were overweight and those with obesity as well as in participants with NAFLD. Differences in sampling, location, and cultural backgrounds might partly explain our findings.

Limitations of the study

There are some limitations in our study. First, the number of participants enrolled in each subgroup was small. Although results depict a clear trend pointing towards the existence of extraction abnormalities (probably flow mediated) and functional liver alterations appearing early in the development of NAFLD, further observations are needed to confirm these data.

Second, the absence of histology and the use of non-invasive diagnostic tools might have generated an underestimation of the ultrasonographic diagnosis of steatosis (*i.e.* unrecognised mild steatosis) and an inaccurate fibrosis assessment by ARFI, a diagnostic technique still not fully validated in NAFLD. A recent

analysis, however, indicated ARFI as a suitable tool in staging liver fibrosis of non-viral origin, mainly in the case of severe fibrosis.[57] By contrast, over- or underestimation of the anatomopathological results is also possible following liver histology.[10,11]

Third, we used a dichotomous split-up of the populations, leading to very heterogeneous categories that cover a whole spectrum of severity. However, logistic regression models considering the whole population of enrolled participants confirmed the increased risk of altered DOB_{15} and $cPDR_{30}$ according to the degree of NAFLD, after adjusting for covariates.

Conclusions

We found that liver dynamic function in response to methacetin demethylation was deranged with respect to increasing body weight and NAFLD, and that NAFLD was also associated with impaired liver function, although tightly associated with obesity and metabolic abnormalities. Our study supports the hypothesis that the interplays between NAFLD, visceral fat accumulation, and adipose tissue dysfunction result in an increased intrahepatic vascular resistance, with flow alterations determining extraction abnormalities during NAFLD development.

The extraction abnormalities (probably flow mediated rather than function mediated) and the functional abnormalities seem to appear early during the development of NAFLD, and should therefore be considered in the initial diagnostic work-up and in the follow-up of these individuals.

Further studies should assess the efficacy of this approach, considering, in particular, possible primary and secondary prevention measures in individuals at high risk for liver and systemic diseases.

Abbreviations

ALT, alanine aminotransferase; ARFI, acoustic radiation force impulse; AST, aspartate aminotransferase; BT, breath test; cPDR, cumulative per cent dose recovery; DOB, delta over baseline; FLI, fatty liver index; GGT, gamma-glutamyl transferase; HOMA, Homeostatic Model Assessment for Insulin Resistance; HRQoL, health-related quality of life; IDF, International Diabetes Federation; KICA, ketoisocaproic acid; MBT, methacetin breath test; MD, Mediterranean diet; MET, metabolic equivalent task; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OR, odds ratio; R-ATPIII, Revised National Cholesterol Education Programme-Adult Treatment Panel III; SF-36, 36-Item Short Form Health Survey Questionnaire; US, ultrasonography; (^{13}C), carbon-13.

Financial support

The present paper originates in the context of the projects FOIE GRAS (#722619) and mtFOIE GRAS (#734719), which have received funding from the European Union's Horizon 2020 research and innovation framework, under the Marie Skłodowska-Curie Grant Agreement (recipients: E.M.-M. and H.S.).

Conflicts of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Clinical study conduction: all authors. Study design: P.P. Wrote the paper, analysed and interpreted the data: E.M.-M. Assessed liver function in participants: E.M.-M., H.S., D.M.D.P. Provided comments and helped with ultrasonographic measurements: V.O.P. Drafted the first version of the paper and finalised the last version: E.M.-M. Performed the full revision of the paper: E.M.-M., D.M.D.P., and P.P. Elaborated the data and revised the paper: A.D.C. and I.G. P.P. had full access to all the data and takes full responsibility for the veracity of the data and the statistical analysis.

Acknowledgements

The authors would like to thank Rosa De Venuto, Paola De Benedictis, Rossella Lenoci, and Eric Mossel for their excellent technical support to this study. The authors also thank Michela Capurso and Enrica Settimo for performing some ultrasonographic measurements.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author, PP, upon reasonable request. The data are not publicly available due to information that could compromise the privacy of research participants.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jhepr.2020.100203.

References

[1] Molina-Molina E, Krawczyk M, Stachowska E, Lammert F, Portincasa P. Non-alcoholic fatty liver disease in non-obese individuals: prevalence, pathogenesis and treatment. Clin Res Hepatol Gastroenterol 2019;43:638–645.
[2] Zhou J, Zhou F, Wang W, Zhang XJ, Ji YX, Zhang P, et al. Epidemiological features of NAFLD from 1999 to 2018 in China. Hepatology 2020;71:1851–1864.
[3] Eslam M, Sanyal AJ, George J, International Consensus Panel Collaborators. MAFLD: a consensus-driven proposed nomenclature for metabolic associated fatty liver disease. Gastroenterology 2020;158:1999–2014.e1.
[4] Ludwig J, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. Mayo Clin Proc 1980;55:434–438.
[5] Ghouri YA, Mian I, Rowe JH. Review of hepatocellular carcinoma: epidemiology, etiology, and carcinogenesis. J Carcinog 2017;16:1.
[6] Younossi ZM. Non-alcoholic fatty liver disease—a global public health perspective. J Hepatol 2019;70:531–544.
[7] Lindenmeyer CC, McCullough AJ. The natural history of nonalcoholic fatty liver disease—an evolving view. Clin Liver Dis 2018;22:11–21.
[8] Assimakopoulos K, Karaivazoglou K, Tsermpini EE, Diamantopoulou G, Triantos C. Quality of life in patients with nonalcoholic fatty liver disease: a systematic review. J Psychosom Res 2018;112:73–80.
[9] Gunn NT, Shiffman ML. The use of liver biopsy in nonalcoholic fatty liver disease: when to biopsy and in whom. Clin Liver Dis 2018;22:109–119.
[10] Regev A, Berho M, Jeffers LJ, Milikowski C, Molina EG, Pyrsopoulos NT, et al. Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. Am J Gastroenterol 2002;97:2614–2618.
[11] Rousselet MC, Michalak S, Dupre F, Croue A, Bedossa P, Saint-Andre JP, et al. Sources of variability in histological scoring of chronic viral hepatitis. Hepatology 2005;41:257–264.
[12] Neuschwander-Tetri BA, Clark JM, Bass NM, Van Natta ML, Unalp-Arida A, Tonascia J, et al. Clinical, laboratory and histological associations in adults with nonalcoholic fatty liver disease. Hepatology 2010;52:913–924.
[13] Gawrieh S, Wilson LA, Cummings OW, Clark JM, Loomba R, Hameed B, et al. Histologic findings of advanced fibrosis and cirrhosis in patients with nonalcoholic fatty liver disease who have normal aminotransferase levels. Am J Gastroenterol 2019;114:1626–1635.
[14] Sviklane L, Olmane E, Dzerve Z, Kupcs K, Pirags V, Sokolovska J. Fatty liver index and hepatic steatosis index for prediction of non-alcoholic fatty liver disease in type 1 diabetes. J Gastroenterol Hepatol 2018;33:270–276.
[15] Schwenzer NF, Springer F, Schraml C, Stefan N, Machann J, Schick F. Non-invasive assessment and quantification of liver steatosis by ultrasound, computed tomography and magnetic resonance. J Hepatol 2009;51:433–445.
[16] Grattagliano I, Lauterburg BH, Palasciano G, Portincasa P. ^{13}C-Breath tests for clinical investigation of liver mitochondrial function. Eur J Clin Invest 2010;40:843–850.

[17] Bonfrate L, Grattagliano I, Palasciano G, Portincasa P. Dynamic carbon 13 breath tests for the study of liver function and gastric emptying. Gastroenterol Rep (Oxf) 2015;3:12–21.
[18] Merkel C, Bolognesi M, Bellon S, Bianco S, Honisch B, Lampe H, et al. Aminopyrine breath test in the prognostic evaluation of patients with cirrhosis. Gut 1992;33:836–842.
[19] Festi D, Capodicasa S, Sandri L, Colaiocco-Ferrante L, Staniscia T, Vitacolonna E, et al. Measurement of hepatic functional mass by means of ^{13}C-methacetin and ^{13}C-phenylalanine breath tests in chronic liver disease: comparison with Child-Pugh score and serum bile acid levels. World J Gastroenterol 2005;11:142–148.
[20] Gorowska-Kowolik K, Chobot A, Kwiecien J. (13)C Methacetin breath test for assessment of microsomal liver function: methodology and clinical application. Gastroenterol Res Pract 2017;2017:7397840.
[21] Lalazar G, Pappo O, Hershcovici T, Hadjaj T, Shubi M, Ohana H, et al. A continuous ^{13}C methacetin breath test for noninvasive assessment of intrahepatic inflammation and fibrosis in patients with chronic HCV infection and normal ALT. J Viral Hepat 2008;15:716–728.
[22] Stravitz RT, Reuben A, Mizrahi M, Lalazar G, Brown K, Gordon SC, et al. Use of the methacetin breath test to classify the risk of cirrhotic complications and mortality in patients evaluated/listed for liver transplantation. J Hepatol 2015;63:1345–1351.
[23] Portincasa P, Grattagliano I, Lauterburg BH, Palmieri VO, Palasciano G, Stellaard F. Liver breath tests non-invasively predict higher stages of non-alcoholic steatohepatitis. Clin Sci (Lond) 2006;111:135–143.
[24] Fierbinteanu-Braticevici C, Plesca DA, Tribus L, Panaitescu E, Braticevici B. The role of C-13-methacetin breath test for the non-invasive evaluation of nonalcoholic fatty liver disease. J Gastrointestin Liver Dis 2013;22:149–156.
[25] Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, et al. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Circulation 2005;112:2735–2752.
[26] Alberti KG, Zimmet P, Shaw J. The metabolic syndrome—a new worldwide definition. Lancet 2005;366:1059–1062.
[27] Minetto MA, Motta G, Gorji NE, Lucini D, Biolo G, Pigozzi F, et al. Reproducibility and validity of the Italian version of the International Physical Activity Questionnaire in obese and diabetic patients. J Endocrinol Invest 2018;41:343–349.
[28] Mendes MA, da Silva I, Ramires V, Reichert F, Martins R, Ferreira R, et al. Metabolic equivalent of task (METs) thresholds as an indicator of physical activity intensity. PLoS One 2018;13:e0200701.
[29] Sofi F, Macchi C, Abbate R, Gensini GF, Casini A. Mediterranean diet and health status: an updated meta-analysis and a proposal for a literature-based adherence score. Public Health Nutr 2014;17:2769–2782.
[30] Marventano S, Mistretta A, Platania A, Galvano F, Grosso G. Reliability and relative validity of a food frequency questionnaire for Italian adults living in Sicily, Southern Italy. Int J Food Sci Nutr 2016;67:857–864.
[31] Ware Jr JE, Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. Med Care 1992;30:473–483.
[32] Kroenke K, Spitzer RL, Williams JB. The PHQ-9: validity of a brief depression severity measure. J Gen Intern Med 2001;16:606–613.
[33] Schneider A, Caspary WF, Saich R, Dietrich CF, Sarrazin C, Kuker W, et al. ^{13}C-methacetin breath test shortened: 2-point-measurements after 15 minutes reliably indicate the presence of liver cirrhosis. J Clin Gastroenterol 2007;41:33–37.
[34] Dinesen L, Caspary WF, Chapman RW, Dietrich CF, Sarrazin C, Braden B. ^{13}C-Methacetin-breath test compared to also noninvasive biochemical blood tests in predicting hepatic fibrosis and cirrhosis in chronic hepatitis C. Dig Liver Dis 2008;40:743–748.
[35] Braden B, Faust D, Sarrazin U, Zeuzem S, Dietrich CF, Caspary WF, et al. ^{13}C-Methacetin breath test as liver function test in patients with chronic hepatitis C virus infection. Aliment Pharmacol Ther 2005;21:179–185.
[36] Holtmeier J, Leuschner M, Schneider A, Leuschner U, Caspary WF, Braden B. ^{13}C-Methacetin and ^{13}C-galactose breath tests can assess restricted liver function even in early stages of primary biliary cirrhosis. Scand J Gastroenterol 2006;41:1336–1341.
[37] Ilan Y. Review article: the assessment of liver function using breath tests. Aliment Pharmacol Ther 2007;26:1293–1302.
[38] Kasicka-Jonderko A, Loska D, Jonderko K, Kaminska M, Blonska-Fajfrowska B. Interference of acute cigarette smoking with [^{13}C]methacetin breath test. Isotopes Environ Health Stud 2011;47:34–41.
[39] Hydzik P, Bielanski W, Ponka M, Wojcicki M, Lubikowski J, Pach J, et al. Usefulness of ^{13}C-methacetin breath test in liver function testing in *Amanita phalloides* poisoning; breast feeding woman case. Clin Toxicol (Phila) 2008;46:1077–1082.
[40] Palmentieri B, de Sio I, La Mura V, Masarone M, Vecchione R, Bruno S, et al. The role of bright liver echo pattern on ultrasound B-mode examination in the diagnosis of liver steatosis. Dig Liver Dis 2006;38:485–489.
[41] Bedogni G, Bellentani S, Miglioli L, Masutti F, Passalacqua M, Castiglione A, et al. The fatty liver index: a simple and accurate predictor of hepatic steatosis in the general population. BMC Gastroenterol 2006;6:33.
[42] Liu H, Fu J, Hong R, Liu L, Li F. Acoustic radiation force impulse elastography for the non-invasive evaluation of hepatic fibrosis in non-alcoholic fatty liver disease patients: a systematic review & meta-analysis. PLoS One 2015;10:e0127782.
[43] Hegazy MA, Samy MA, Tawfik A, Naguib MM, Ezzat A, Behiry ME. Abdominal subcutaneous fat thickness and homeostasis model assessment of insulin resistance as simple predictors of nonalcoholic steatohepatitis. Diabetes Metab Syndr Obes 2019;12:1105–1111.
[44] Hamagawa K, Matsumura Y, Kubo T, Hayato K, Okawa M, Tanioka K, et al. Abdominal visceral fat thickness measured by ultrasonography predicts the presence and severity of coronary artery disease. Ultrasound Med Biol 2010;36:1769–1775.
[45] Brunetti ND, Lanzone S, Dellegrottaglie G, Di Giuseppe G, De Gennaro L, Novielli V, et al. The CAPITAL study (CArdiovascular prevention wIth Telecardiology in ApuLia): preliminary results. J Cardiovasc Med 2016;17:455–461.
[46] O'Neill S, O'Driscoll L. Metabolic syndrome: a closer look at the growing epidemic and its associated pathologies. Obes Rev 2015;16:1–12.
[47] Vecchie A, Dallegri F, Carbone F, Bonaventura A, Liberale L, Portincasa P, et al. Obesity phenotypes and their paradoxical association with cardiovascular diseases. Eur J Intern Med 2018;48:6–17.
[48] Moran S, Mina A, Duque X, Ortiz-Olvera N, Rodriguez-Leal G, Alfredo Sierra-Ramirez J, et al. The utility of the (13)C-methacetin breath test in predicting the long-term survival of patients with decompensated cirrhosis. J Breath Res 2017;11:036011.
[49] Klatt S, Taut C, Mayer D, Adler G, Beckh K. Evaluation of the ^{13}C-methacetin breath test for quantitative liver function testing. Z Gastroenterol 1997;35:609–614.
[50] Motamed N, Sohrabi M, Ajdarkosh H, Hemmasi G, Maadi M, Sayeedian FS, et al. Fatty liver index vs waist circumference for predicting non-alcoholic fatty liver disease. World J Gastroenterol 2016;22:3023–3030.
[51] Clemente AP, Netto BD, de Carvalho-Ferreira JP, da Silveira Campos RM, de Piano Ganen A, Tock L, et al. Waist circumference as a marker for screening nonalcoholic fatty liver disease in obese adolescents. Rev Paul Pediatr 2016;34:47–55.
[52] Pasarin M, Abraldes JG, Liguori E, Kok B, La Mura V. Intrahepatic vascular changes in non-alcoholic fatty liver disease: potential role of insulin-resistance and endothelial dysfunction. World J Gastroenterol 2017;23:6777–6787.
[53] Francque S, Laleman W, Verbeke L, Van Steenkiste C, Casteleyn C, Kwanten W, et al. Increased intrahepatic resistance in severe steatosis: endothelial dysfunction, vasoconstrictor overproduction and altered microvascular architecture. Lab Invest 2012;92:1428–1439.
[54] van der Graaff D, Kwanten WJ, Francque SM. The potential role of vascular alterations and subsequent impaired liver blood flow and hepatic hypoxia in the pathophysiology of non-alcoholic steatohepatitis. Med Hypotheses 2019;122:188–197.
[55] Francque S, Verrijken A, Mertens I, Hubens G, Van Marck E, Pelckmans P, et al. Noncirrhotic human nonalcoholic fatty liver disease induces portal hypertension in relation to the histological degree of steatosis. Eur J Gastroenterol Hepatol 2010;22:1449–1457.
[56] Lefere S, Devisscher L, Geerts A. Angiogenesis in the progression of non-alcoholic fatty liver disease. Acta Gastroenterol Belg 2020;83:301–307.
[57] Lin Y, Li H, Jin C, Wang H, Jiang B. The diagnostic accuracy of liver fibrosis in non-viral liver diseases using acoustic radiation force impulse elastography: a systematic review and meta-analysis. PLoS One 2020;15:e0227358.
[58] Varela JE, Hinojosa M, Nguyen N. Correlations between intra-abdominal pressure and obesity-related co-morbidities. Surg Obes Relat Dis 2009;5:524–528.
[59] Maximos M, Bril F, Portillo Sanchez P, Lomonaco R, Orsak B, Biernacki D, et al. The role of liver fat and insulin resistance as determinants of plasma aminotransferase elevation in nonalcoholic fatty liver disease. Hepatology 2015;61:153–160.
[60] Chen Z, Qin H, Qiu S, Chen G, Chen Y. Correlation of triglyceride to high-density lipoprotein cholesterol ratio with nonalcoholic fatty liver disease

among the non-obese Chinese population with normal blood lipid levels: a retrospective cohort research. Lipids Health Dis 2019;18:162.
[61] Ren XY, Shi D, Ding J, Cheng ZY, Li HY, Li JS, et al. Total cholesterol to high-density lipoprotein cholesterol ratio is a significant predictor of nonalcoholic fatty liver: Jinchang cohort study. Lipids Health Dis 2019;18:47.
[62] Tirosh O. Hypoxic signaling and cholesterol lipotoxicity in fatty liver disease progression. Oxid Med Cell Longev 2018;2018:2548154.
[63] Ho CM, Ho SL, Jeng YM, Lai YS, Chen YH, Lu SC, et al. Accumulation of free cholesterol and oxidized low-density lipoprotein is associated with portal inflammation and fibrosis in nonalcoholic fatty liver disease. J Inflamm (Lond) 2019;16:7.
[64] Gastaldelli A, Cusi K. From NASH to diabetes and from diabetes to NASH: Mechanisms and treatment options. JHEP Rep 2019;1:312–328.
[65] Moosavian SP, Arab A, Paknahad Z. The effect of a Mediterranean diet on metabolic parameters in patients with non-alcoholic fatty liver disease: a systematic review of randomized controlled trials. Clin Nutr ESPEN 2020;35:40–46.
[66] Smart NA, King N, McFarlane JR, Graham PL, Dieberg G. Effect of exercise training on liver function in adults who are overweight or exhibit fatty liver disease: a systematic review and meta-analysis. Br J Sports Med 2018;52:834–843.
[67] David K, Kowdley KV, Unalp A, Kanwal F, Brunt EM, Schwimmer JB. Quality of life in adults with nonalcoholic fatty liver disease: baseline data from the nonalcoholic steatohepatitis clinical research network. Hepatology 2009;49:1904–1912.
[68] Kim D, Yoo ER, Li AA, Tighe SP, Cholankeril G, Harrison SA, et al. Depression is associated with non-alcoholic fatty liver disease among adults in the United States. Aliment Pharmacol Ther 2019;50:590–598.