The Role of $^{13}$C-Methacetin Breath Test for the Non-Invasive Evaluation of Nonalcoholic Fatty Liver Disease

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ABSTRACT

Background & Aims: Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in many parts of the world. The $^{13}$C-methacetin breath test (MBT), a microsomal liver function test, enables quantitative evaluation of cytochrome P450-dependent liver function involved in NAFLD pathogenesis. The aim of our study was to evaluate the efficacy of MBT in differentiating patients with non-alcoholic steatohepatitis (NASH) from patients with simple steatosis (SS) and its ability to predict significant fibrosis in NAFLD patients.

Methods: We performed MBT in 64 patients with histologically proven NAFLD (ranging from SS to severe steatohepatitis) and in 20 healthy controls. Brunt scoring system for histological evaluation of NAFLD served as a reference. The correlation between MBT parameters and liver biopsy was tested using Spearman's coefficient. The overall validity was measured using the area under the receiver operating characteristic curve (AUROC) with 95%CI.

Results. $^{13}$C- MBT is a good tool for identifying patients with histologically proven NASH, with an AUROC of 0.824, 95% CI (0.723-0.926), a sensitivity of 95% and a specificity of 74%. The diagnosis accuracy of $^{13}$C- MBT for significant fibrosis (F≥2) has a validity of 91% (95% CI, AUROC = 0.830 – 0.989) with higher sensitivity (90%) and specificity (81%). $^{13}$C- MBT values predicted better F3 or F4 fibrosis (AUROC were 0.936 and 0.973)

Conclusion. Due to the impairment of microsomal function which occurs in NAFLD, $^{13}$C- MBT could be a reliable diagnostic and follow-up test for NAFLD patients.

Key words: $^{13}$C-methacetin breath test (MBT) – cytochrome P450 – nonalcoholic fatty liver disease (NAFLD) – nonalcoholic steatohepatitis (NASH) – liver fibrosis

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in many parts of the world. Its prevalence continues to rise, currently affecting 20 to 30% of adults [1] and 10% of children [2] and threatens to become a serious public health problem. Non-alcoholic fatty liver disease represents a wide spectrum of conditions ranging from fatty liver, which in general follows a benign non progressive clinical course, to steatohepatitis (NASH), a more serious form of NAFLD that may progress to cirrhosis and end-stage liver disease. Liver biopsy remains the gold standard to stage NAFLD, but it does have limitations that make it an unfeasible approach in all NAFLD subjects: it is costly, invasive, and has complications requiring hospitalization in 1-3% of cases and a procedural mortality of 0.01% [3]. Furthermore, liver biopsy is subject to intra- and inter-observer reading variability, and to sample inadequacy or variability due to uneven disease distribution, which lead to missing a diagnosis of NASH in up to 27% of cases [4]. Therefore, the development of non-invasive tests for identifying patients with NASH before the onset of advanced fibrosis is the goal of diagnostic procedures and has become an active area of research [5]. Dynamic breath tests can detect specific alterations in metabolic pathways. The $^{13}$C-methacetin breath test (MBT), a microsomal liver function test, enables quantitative evaluation of cytochrome P450-dependent liver function, involved in the development of NASH. The aim of our study was to evaluate the efficacy of MBT in differentiating patients with simple steatosis (SS) from NASH patients and its ability to predict significant fibrosis in NAFLD patients.
METHODS

Study population
We evaluated 90 patients with histologically proven NAFLD diagnosed at the University Hospital Bucharest between 2007-2010. Liver biopsy was performed for abnormal liver function tests and suspected NAFLD at grey scale ultrasonography. The patients with other liver disease etiologies: significant alcohol abuse, evidence of hepatitis B and C, drug induced liver disease or other specific liver diseases (hemochromatosis, α1 antitripsin deficiency, Wilson’s disease, autoimmune liver disease) were excluded. None of the patients had a clinical history of liver decompensation (ascites, bleeding from varices, and encephalopathy). We also excluded patients with biopsy specimens’ length < 20 mm including those with biopsies of <8 portal tracts (11 patients). Patients taking drugs with modulating capacity on P450 cytochrome activity, heavy smokers and those with chronic liver and lung diseases were also excluded (15 patients).

A total of 20 healthy volunteers served as controls. No one had a history of alcohol abuse, smoking, medication intake or previous lung and liver diseases. Routine liver tests and ultrasonographic abdominal evaluation were normal.

The study was performed in accordance with the Declaration of Human Rights (Helsinki) and was approved by the local Ethics Committee. A written informed consent was obtained from each patient.

Clinical evaluation
All patients underwent comprehensive assessment including history and physical examination. Height, weight, and waist circumference were determined. Body mass index (BMI) was calculated (kg/m²) as weight (kg) divided by height (m²). Overweight or the degree of obesity was established using BMI cut-off points of 25-29.9, 30-34.9, 35-39.9 and >40 kg/m², respectively. Visceral obesity was identified by measuring waist circumference (WC) at the midpoint between the lower border of the rib cage and the iliac crest. Systolic/diastolic blood pressure was defined as the mean of the second and third reading of three consecutive blood pressure measurements.

Biochemical evaluation
Blood samples were obtained under fasting conditions and the following tests were performed using standard laboratory methods: alanin aminotransferase (ALT), aspartate aminotransferase (AST), serum albumin, γ-glutamyl transeptidase (γGT), total bilirubin, alkaline phosphatase, international normalized ratio (INR), glucose, cholesterol, triglycerides, uric acid, serum concentration of iron and ferritin. All were measured by using Dade Behring reactants and the Dimension RXL analyzer (Dade Behring, FL, USA). Insulin resistance was calculated according to the HOMA index (HOMeostatic Metabolic Assessment). C-reactive protein (CRP) values were determined by the ELISA test. Oxidative stress was evaluated by assessing the serum lipid peroxidation product malondialdehyde (MDA), and serum glutathione (GTH).

Ultrasound evaluation

All the patients were investigated by ultrasonography (US) using Acuson S2000 Siemens machine. Spleen volume was estimated by spleen longitudinal diameter (SLD). We used the maximum length obtained between the two poles of the spleen and the measurements were performed by postero-lateral scanning. The classification of “bright liver” or hepatic steatosis (HS) was based on a four-point scale of hyperechogenity: 0 = absent, 1 = light, 2 = moderate, 3 = severe, according to the difference between the densities of the liver and the right kidney.

Histological evaluation
All the enrolled patients underwent histological assessment by percutaneous liver biopsy. This was performed by senior physicians, using the Menghini technique with a 1.4-mm-diameter needle (Hepafix; Braun, Germany). All biopsy specimens were analysed by an expert pathologist (20 year experience) blinded to the patients’ clinical results. The length of each liver biopsy was established in millimetres and the number of portal tracts was counted. Only liver fragments of at least 2.0 cm in length, that included 8 portal tracts were considered for histological assessment. The diagnosis of NASH was based on the criteria of Brunt et al (Brunt et al. 1999), modified by Kleiner et al (2005). The stage of fibrosis was scored based on a five-point scale, as follows: stage 0, absence of fibrosis; stage 1, perisinusoidal or portal fibrosis; stage 2, perisinusoidal and portal periportal fibrosis; stage 3, septal or bridging fibrosis; and stage 4, cirrhosis. The severity of steatosis was graded from 1 to 3, according to the percentage of cells with fatty droplets (degree 1: 10%-33%; 2: 33%-66%; and 3: >66%).

13C-methacetin breath test
We performed the 13C-MBT in 64 patients with histologically proven NAFLD and in 20 healthy controls. 13C-methacetin (N-(4-13Cmethyleneoxyphenyl) acetamide) is metabolized by single N-dealkylation to 13CO2 and acetaminophen in the liver via microsomal mixed function oxidative system. The 13CO2 produced is exhaled. In a well structured 13C – breath test, 13C/12C isotope ratios are measured by the IRIS-analyser (Infra Red Isotope Analyser, Wagner – Analysetechnik, Bremen, Germany) in automated mode in the breath samples which have been blown into breath bags by patient. The percentage of 13C exhaled was calculated assuming a CO2 production rate of 5mmol/min. The results were expressed as the percentage of the administered dose exhaled/h (% 13C-dose/h) and the cumulative percentage (% of the administered dose of 13C recovered over time (% CUM).

The breath test was performed after an overnight fast and during the test any food consumption and physical activity were prohibited. After collecting the first control sample of air into a bag, each subject ingested 75 mg methacetin labeled with stable, non-radioactive isotope 13C solved in 200 ml water. Breath samples were collected at baseline and every ten minutes over 1 hour after ingestion of the substrate. IRIS-data system
acquired the isotope data, calculated from patient's body height his physiologic CO2 production and provided the graphs for the metabolisation speed (Kinetics: 13C-dose/h: percentage of the administered dose exhaled/h) and for the metabolisation capacity (Cum.dose: percentage of the administered dose exhaled at each time point). Standardized graphs allow the comparison of the patient's metabolisation parameters with the 95% confidence limits of collective Normal Controls over the time span of the breath test.

**Statistical analysis**

Results are presented as the mean ± SD, for a Gaussian distribution and as the median and 25th- to 75th-percentile values for a non-Gaussian distribution. We used one way ANOVA, Kruskal-Wallis and multiple comparison post hoc tests to compare MBT results between the NASH group, the SS group and the control group. The correlations between the 13C –methacetin breath test results, clinical and biological characteristics were tested using Spearman's or Pearson's correlation coefficients. The overall validity was measured using the area under the receiver operating characteristic curve (AUROC) with 95% CI. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated. The optimal cut-off was chosen at the highest left point on the curve. For all tests, significance was achieved at \( p < 0.05 \). Statistical tests were performed using SPSS software (version 15).

**RESULTS**

Sixty four patients (test group) fulfilling inclusion criteria and 20 healthy participants (control group) were included in the final analysis. According to the histological characteristics of the liver biopsy, the test group was divided in two subgroups: subgroup 1 (43 patients) consisted of patients with histologically proven NASH and subgroup 2 (21 patients) consisted of patients with simple steatosis (SS). Demographic, clinical and biochemical characteristics of the patients included in the study are shown in Table I.

All NAFLD patients had significantly higher serum levels of transaminases, cholesterol, triglyceride, glucose and γGT than control subjects. Values of CRP and SLD were higher in patients with NASH compared to patients with SS.

The distribution of the patients with NAFLD according to the histological characteristics are presented in Table II.

The efficacy of 13C-MBT to identify patients with NASH

Patients with SS had similar values of MBT regarding the kinetics (13C-dose/h) and the metabolisation capacity (Cum. dose) as the control group. The peak 13C exhalation appeared at 10 min in both these groups without differences regarding the dose metabolized at peak (32.6 ±4.20 vs. 35.8 ± 1.76, \( p=NS \)). According to these findings, the 13C-dose at 10 min was selected as the cut-off parameter. Unlike patients with SS, NASH patients presented a peak shift toward 30 min with

### Table I. Patients characteristics in the NASH group, the Simple Steatosis group and Control group (Average ±SD/Median )

<table>
<thead>
<tr>
<th></th>
<th>NASH (N=43)</th>
<th>Simple steatosis (N=21)</th>
<th>Control (N=20)</th>
<th>( P_value )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>43</td>
<td>21</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Sex M/F</td>
<td>20/23</td>
<td>8/13</td>
<td>8/12</td>
<td>0.7805***</td>
</tr>
<tr>
<td>Age (years)</td>
<td>51.0 [46.0,57.0]</td>
<td>47.0 [39.0,54.0]</td>
<td>45.0 [37.5,52.5]</td>
<td>0.0275**</td>
</tr>
<tr>
<td>WC (cm) male</td>
<td>97.05</td>
<td>97.12±2.74</td>
<td>89.87±3.79</td>
<td>0.0171*</td>
</tr>
<tr>
<td>WC (cm) female</td>
<td>89.73±10.8</td>
<td>86.46±9.4</td>
<td>74.41±6.3</td>
<td>0.003*</td>
</tr>
<tr>
<td>BMI (kg/m²) male</td>
<td>29.5 [28.5,31.7]</td>
<td>29.5 [27.2,30.5]</td>
<td>26.9 [26.3,27.6]</td>
<td>0.0031**</td>
</tr>
<tr>
<td>BMI (kg/m²) female</td>
<td>28.98±2.83</td>
<td>29.03±2.57</td>
<td>25.40 ±1.56</td>
<td>0.0004*</td>
</tr>
<tr>
<td>Spleen (mm)</td>
<td>117.59 ±13.67</td>
<td>94.46±14.32</td>
<td>89.75±9.90</td>
<td>0.0000*</td>
</tr>
<tr>
<td>Glucose mg/dl</td>
<td>123.0 [98.0,132.0]</td>
<td>103.0 [89.0,119.0]</td>
<td>83.0 [78.0,90.5]</td>
<td>0.0000**</td>
</tr>
<tr>
<td>HOMA</td>
<td>3.10 [2.70,3.50]</td>
<td>3.00 [2.90,3.00]</td>
<td>-</td>
<td>0.2143**</td>
</tr>
<tr>
<td>Triglyceride mg/dl</td>
<td>211 [169,240]</td>
<td>172 [166,189]</td>
<td>95.5 [88.5,129]</td>
<td>0.0000**</td>
</tr>
<tr>
<td>HDL-CHOL mg/dl</td>
<td>35.62±5.90</td>
<td>37.45± 6.32</td>
<td>48.70± 4.54</td>
<td>0.0000**</td>
</tr>
<tr>
<td>ALT UI/l</td>
<td>92 [69.0,107.0]</td>
<td>67.0 [52.0,86.0]</td>
<td>42.0 [29.5,54.5]</td>
<td>0.0000**</td>
</tr>
<tr>
<td>AST UI/l</td>
<td>88.0 [77.0,94.0]</td>
<td>81.0 [64.0,96.0]</td>
<td>22.5 [20.0,29.0]</td>
<td>0.0000**</td>
</tr>
<tr>
<td>Albumin g/dl</td>
<td>3.60 ±0.60</td>
<td>3.92 ± 0.46</td>
<td>5.11 ± 0.53</td>
<td>0.0000*</td>
</tr>
<tr>
<td>INR</td>
<td>1.05±0.13</td>
<td>0.99±0.13</td>
<td>0.915±0.10</td>
<td>0.0015*</td>
</tr>
<tr>
<td>γGT UI/l</td>
<td>108.0 [88.0,127.0]</td>
<td>85.0 [72.0,98.0]</td>
<td>62.5 [55.0,72.0]</td>
<td>0.0000**</td>
</tr>
<tr>
<td>CRP UI/ml</td>
<td>5.25±1.82</td>
<td>2.86± 2.14</td>
<td>-</td>
<td>0.0000*</td>
</tr>
<tr>
<td>MDH nmol/nl</td>
<td>2.48±1.09</td>
<td>1.37± 0.98</td>
<td>-</td>
<td>0.0002*</td>
</tr>
<tr>
<td>GTH μmol/l</td>
<td>4.32± 0.80</td>
<td>5.14±0.66</td>
<td>-</td>
<td>0.0002*</td>
</tr>
</tbody>
</table>

\( P < 0.05 \) (ANOVA or Kruskal-Wallis with multiple comparison post hoc tests).* ANOVA/ **Kruskall-Wallis/ *** Chi-square.

WC= Waist circumference, BMI = Body mass Index, HOMA = Homeostatic Metabolic Assessment, HDL–CHOL = High Density Lipoprotein- Cholesterol, ALT = alanine aminotransferase, AST = aspartate aminotransferase, INR = international normalized ratio, γGT = γ-glutamyl transferase, CRP= C- reactive protein, MDA = malondialdehyde, GTH = glutathione
a moderate decrease of the dose metabolized at peak (28.5 ± 14.01, p<0.001). The characteristic curves for kinetics of methacetin metabolism in NAFLD patients and in controls are shown in Fig. 1. The shape of the curve reflects the dynamics of the methacetin metabolism.

In the control and SS groups the kinetics curves were similar in shape: the peak time occurred at about 10 min after administration of methacetin and then the curves lowered abruptly. The shape is obviously distinct in the NASH group. The peak occurred later, at about 30 min after administration of methacetin and the declining phase was much slower.

Compared with controls and patients with SS, patients with NASH had a lower capacity to metabolise methacetin, expressed as cumulative recovery of $^{13}$CO$_2$ in breath (CUM). The higher difference threshold between patients with NASH and those with steatosis was noted for CUM at 60 min after methacetin ingestion (13.1±5.4 vs. 20.6±1.84, p<0.001).

There were no significant differences between the group with steatosis and the control group regarding the values of CUM at 60 min in the $^{13}$C-MBT (20.6±1.84 vs 22.5±1.15, p=NS). The diagrams for the capacity of methacetin metabolism in NAFLD patients and in controls are shown in Fig. 2. The shape of the curve provides information about the global liver capacity to metabolize methacetin.

Both parameters of $^{13}$C-MBT: $^{13}$C-Dose at 10 min and $^{13}$C-CUM at 60 min, have a similar accuracy for detecting NASH with an AUROC of 0.822 and 0.824, respectively.

The optimal cut-off values chosen to maximize the discriminatory ability of $^{13}$C-MBT to identify patients with NASH, the validity and predictive values are presented in Table III.

Receiver operating characteristic analysis of the $^{13}$C-MBT is shown in Fig. 3.

The role of $^{13}$C-MBT in predicting histological stage of fibrosis

The $^{13}$C-MBT results were significantly correlated (Spearman’s coefficient) with histological fibrosis score ($r = -0.763$ for Dose 10, $r = -0.768$ for CUM 60, p<0.001), weakly correlated with inflammation ($r = -0.348$, p=0.005 for Dose 10 and for CUM 60 $r = -0.463$, p<0.001) and were not correlated with steatosis (p=0.125). Significant correlations (Pearson’s coefficient) were obtained between $^{13}$C-MBT results and SLD ($r = -0.731$ for Dose at 10 min and for CUM at 60 min, $r = -0.763$, p<0.001) and with CRP values ($r = -0.429$ for Dose at 10 min and $r = -0.505$ for CUM at 60 min, p<0.001).

The results of $^{13}$C-MBT test were related to the histological fibrosis staging system. The cut-off values showing the best sensitivity and specificity and the accuracy of $^{13}$C-MBT for each stage of fibrosis are presented in Table IV. The AUROC analysis of the $^{13}$C-MBT results (Fig. 4) revealed a cut-off

![Fig. 1. Mean percentage of $^{13}$CO$_2$ exhalation at each time point expressed as % $^{13}$C-dose/h in NAFLD patients and in controls. % $^{13}$C-dose/h = Percentage of the administered dose exhaled/h; NAFLD= Nonalcoholic fatty liver disease.](image1)

![Fig. 2. Mean cumulative recovery in the MBT of NAFLD patients and controls. MBT= Methacetin breath test; Cumulative recovery (%): the cumulative percentage (%) of the administered dose of $^{13}$C recovered over time.](image2)

![Fig. 3. Receiver-operating characteristics analysis for the differentiation between patients with NASH and patients with steatosis using the Dose at 10 min and CUM at 60 min as parameters of the $^{13}$C-methacetin breath test. NASH = Nonalcoholic steatohepatitis.](image3)
Table III. Discriminative cut-off values of $^{13}$C-MBT to identify patients with non-alcoholic steatohepatitis (NASH) from those with simple steatosis (SS)

<table>
<thead>
<tr>
<th>Stage</th>
<th>AUC (95% CI)</th>
<th>Cut-off % $^{13}$C</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NASH/SS</td>
<td>0.822</td>
<td>27.65</td>
<td>95.2%</td>
<td>76.7%</td>
<td>66.67%</td>
<td>97.06%</td>
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<tr>
<td></td>
<td>(0.719-0.924)</td>
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</table>

**CUM at 60 min**

<table>
<thead>
<tr>
<th>Stage</th>
<th>AUC (95% CI)</th>
<th>Cut-off % $^{13}$C</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>VPP</th>
<th>VPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>NASH/SS</td>
<td>0.824</td>
<td>17.15</td>
<td>95.2%</td>
<td>74.4%</td>
<td>64.52%</td>
<td>96.97%</td>
</tr>
<tr>
<td></td>
<td>(0.723-0.926)</td>
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Dose at 10 min: percentage of the administered dose exhaled/h at 10 min; CUM at 60 min: cumulative percentage (%) of the administered dose of $^{13}$C recovered at 60 min. PPV= positive predictive value; NPV negative predictive value.

The $^{13}$C-MBT predicted even better the presence of advanced fibrosis (F≥3): Dose at 10 min (AUROC = 92.9%, 95% CI = 0.837-1) and CUM at 60 min (AUROC = 91.9%, 95% CI = 0.868-1). The same high validity was maintained for cirrhosis prediction: Dose at 10 min; AUROC = 96.5%, 95% CI = 0.919-1, CUM at 60 min (AUROC = 97.3% (0.935-1).

**DISCUSSION**

Although considered the gold standard for assessment of liver fibrosis, liver biopsy has its limitations, including inter-observer variability, sampling error and risks for complications. Despite extensive investigations, currently available tools for differentiating the major NAFLD phenotypes are not yet ready for clinical practice.

In patients with chronic liver disease, quantitative testing of liver function is critical for monitoring disease progression, predicting the prognosis and choosing therapeutic strategies. Breath tests are based on the oral administration of a carbon isotope-labeled substrate, followed by the analysis of labeled CO₂ in expired air [7, 8]. Among the various substrates utilized, methacetin, a derivate of phenacetin, undergoes demethylation/decarboxylation through the hepatic mixed oxidase system using CYP2E1 and CYP1A2 to produce acetaminophen and

Table IV. Discriminative cut-off values of $^{13}$C-MBT for predicting fibrosis in patients with nonalcoholic fatty liver disease (NAFLD) histologically proven

<table>
<thead>
<tr>
<th>Fibrosis</th>
<th>AUC (95% CI)</th>
<th>Cut-off</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>VPP</th>
<th>VPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>F=1</td>
<td>0.822</td>
<td>29.75</td>
<td>85.7%</td>
<td>76.7%</td>
<td>64.29%</td>
<td>91.67%</td>
</tr>
<tr>
<td></td>
<td>(0.719-0.924)</td>
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<tr>
<td>F=2</td>
<td>0.910</td>
<td>19.00</td>
<td>90.3%</td>
<td>81.1%</td>
<td>82.35%</td>
<td>90.00%</td>
</tr>
<tr>
<td></td>
<td>(0.830-0.989)</td>
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<tr>
<td>F=3</td>
<td>0.923</td>
<td>16.40</td>
<td>92.9%</td>
<td>86.4%</td>
<td>92.86%</td>
<td>86.36%</td>
</tr>
<tr>
<td></td>
<td>(0.837 - 1.000)</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>F=4</td>
<td>0.965</td>
<td>11.95</td>
<td>96.2%</td>
<td>91.7%</td>
<td>98.04%</td>
<td>84.62%</td>
</tr>
<tr>
<td></td>
<td>(0.919 - 1.000)</td>
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</tbody>
</table>

**CUM at 60 min**

<table>
<thead>
<tr>
<th>Fibrosis</th>
<th>AUC (95% CI)</th>
<th>Cut-off</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>VPP</th>
<th>VPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>F=1</td>
<td>0.824</td>
<td>18.40</td>
<td>85.7%</td>
<td>74.4%</td>
<td>62.07%</td>
<td>91.43%</td>
</tr>
<tr>
<td></td>
<td>(0.723 - 0.926)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F=2</td>
<td>0.900</td>
<td>15.25</td>
<td>91.1%</td>
<td>81.8%</td>
<td>83.06%</td>
<td>90.11%</td>
</tr>
<tr>
<td></td>
<td>(0.820 - 0.980)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F=3</td>
<td>0.936</td>
<td>13.40</td>
<td>91.9%</td>
<td>84.4%</td>
<td>92.95%</td>
<td>87.26%</td>
</tr>
<tr>
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<td>(0.868 - 1.000)</td>
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<tr>
<td>F=4</td>
<td>0.973</td>
<td>10.55</td>
<td>92.3%</td>
<td>91.7%</td>
<td>97.96%</td>
<td>73.33%</td>
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<td></td>
<td>(0.935 - 1.000)</td>
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Dose at 10 min: percentage of the administered dose exhaled/h at 10 min; CUM at 60 min: cumulative percentage (%) of the administered dose of $^{13}$C recovered at 60 min. PPV= positive predictive value; NPV negative predictive value.
CO₂. Rapid metabolisation in normal subjects, extensive first pass clearance, linear increase of extraction rate of CO₂ with increased dose and lack of any toxicity make methacetin the substrate providing the best kinetics and very good quantitative information for liver function [9].

The present study evaluates the relationship between histological features of NAFLD phenotypes and the ¹³C-MBT, used to explore specific liver microsomal function.

Among the patients with NAFLD, patients with NASH have a higher likelihood of progression to cirrhosis, hepatocellular carcinoma and end-stage liver disease [10, 11]. Therefore, the main target of our study was to assess the discriminatory ability of the ¹³C-MBT between patients with NASH and those with SS. Regarding this point, our study performed on 64 patients with NAFLD showed that ¹³C-Dose 10 and ¹³C-CUM 60 were reliable parameters for the ¹³C-MBT in ROC analysis when compared with liver histology (95% sensitivity and 74-76% specificity). They were also the best parameters for comparison between the groups.

Our study found that the shape of the metacethin kinetics curve was obviously distinct in the NASH group as compared to the control or SS group. The delay of the peak time dose on the diagram probably indicates the disturbance of transport function that occurred with NAFLD progression toward NASH.

The ROC area is a reliable measure to quantify the discriminative power of a diagnostic model; a value >0.8 was considered to represent good discrimination [12]. The present study demonstrated that ¹³C-MBT had a good diagnostic power in identifying patients with NASH (AUC: 0.822 and 0.824).

Sequential studies were performed over the years using ¹³C-methacetin substrate. Most of these studies correlated the ¹³C-MBT values with histologically proven fibrosis stages in patients with chronic liver diseases, especially those of viral etiology. Analysis of the published studies demonstrated that the ¹³C-MBT is reliable and accurate in estimating the severity of liver cirrhosis [13-17]. Dinesen et al, comparing the diagnostic accuracy of ¹³C-MBT with other non-invasive tools, found that MBT was more reliable in predicting advanced fibrosis and cirrhosis in patients with chronic hepatitis than APRI, AAR, and Fibro index [18].

On the other hand, a study of 77 patients that evaluated the diagnostic value of the MBT in various stages of chronic liver diseases showed that the ¹³C-MBT failed to discriminate between different fibrosis stages but accurately indicated advanced cirrhosis [19].

Lalazar et al, using a continuous MBT system based on molecular correlation spectroscopy, concluded that the breath device is a valuable tool for inflammation and fibrosis assessment in patients with chronic HCV infection and normal aminotransferases [20].

Few studies investigated the role of breath tests in NAFLD. In a study conducted on 39 hypertransaminasemic patients with histologically proven NAFLD, the authors evaluated the enzymatic and metabolic liver function using two breath tests: ¹³C-MBT (for microsomal function) and ¹³C-ketoisocaproate (for mitochondrial function). Contrary to our results, they concluded that ¹³C-MBT was not impaired by NASH while ¹³C-ketoisocaproate correlated with NAFLD severity and proved to be a useful test for NASH diagnosis (90% PPV and 73% NPV) [21]. In their study, the methacetin demethylation was higher in patients with NASH as compared to controls, reflecting an increase of the microsomal metabolic activity. Possible reasons for these different results could be the varied distribution among the subjects in these studies. In the study of Portincasa et al, the NAFLD patients with advanced fibrosis were excluded, whereas in our study 34% of the patients presented severe fibrosis and cirrhosis. As ¹³C-MBT is influenced by impaired blood flow and hepatocyte necrosis, which occur in cirrhosis, this could explain the different data [22].

Insulin resistance, oxidative stress, and an inflammatory cascade are believed to play an integrative role in the pathogenesis and progression of NAFLD. Among other biomarkers, high levels of CRP and spleen enlargement were strongly associated with NASH diagnosis [23, 24]. Regarding the relationships between the MBT values and the inflammatory status, we found a significant correlation between the kinetics and capacity of methacetin metabolism values and indices of low grade of chronic inflammation: SLD (r = - .763) and CRP (r = - .505).

Assessment of significant fibrosis is important in NAFLD for deciding the treatment and predicting prognosis. Therefore, a second point of interest was to evaluate the efficacy of ¹³C-MBT in detecting significant fibrosis.

Our study shows that ¹³C-MBT is a reliable, non-invasive tool, that can predict significant fibrosis associated with detrimental effect on liver function, the real determinant of the NAFLD patients’ prognosis. An AUROC value of 0.910 (0.830-0.989) and 0.900 (0.820-0.980) were obtained using the two best parameters, ¹³C-Dose 10 and ¹³C-CUM 60 for the diagnosis of significant fibrosis (≥ F2), with a cut-off value of 19.00% ¹³C-Dose at 10 min and 15.25 ¹³C-CUM 60, respectively. Considering the increased values of specificity and specificity (90% and 81%), the MBT test could be of great help in the evaluation and especially follow-up of patients with NAFLD. The same high validity was maintained as in predicting advanced fibrosis (F≥3) and cirrhosis (F=4). For the diagnosis of cirrhosis in NAFLD patients, ¹³C-MBT values delimited an AUROC of 0.965 (0.919 - 1.000; p<0.001) for Dose 10 and of 0.973 (0.935 – 1.000; p<0.001) for CUM 60.

A series of studies performed over the years also showed that increasing degrees of liver fibrosis are accompanied by concomitant modifications of breath tests results [25-27]. Miele et al performed ¹³C-Octanoate Breath Test (¹³C-OBT) in patients with NAFLD and demonstrated the relationship between the presence of fibrosis and the impairment of liver function, expressed by lower ¹³C-OBT results than those of controls [25]. Park et al showed that the ¹³Caffeine Breath Test, another test for microsomal function, reflected the extent of hepatic fibrosis in NAFLD and was an independent predictor of significant fibrosis in these patients [26]. Another study that used ¹³C-MBT demonstrated a significant correlation between the breath tests’ parameters and NASH activity and also advanced fibrosis [27].

Systemic caffeine clearance, considered the gold standard for phenotyping cytochrome P4501A2 has been recommended for the non-invasive assessment of liver function in chronic liver disease. Tarantino et al found a simple and reliable...
alternative to this method, based on the total overnight salivary caffeine assessment (TOSCA) determined by using a single-point concentration of salivary caffeine, after an overnight period of abstinence. They concluded that TOSCA is a reliable test for evaluating liver function and it can also differentiate between cirrhosis type, such as viral and cryptogenic (likely metabolic) cirrhosis [28].

One limitation of our study is the relatively small number of patients due to the refusal of many invited participants to undergo liver biopsy. Another important problem regarding the use of any tool to discriminate SS from NASH is related to the inner pathogenetic mechanisms of NAFLD. Even though steatosis is a benign condition and NASH a progressive one, the basic mechanisms of both entities seem to be the same. This is supported by the study of Tarantino et al, who reported similar levels of transforming growth factor-β1 in the serum of patients with SS and those with NASH [29].

However, we consider that our study may provide a non-invasive approach to the functional alterations secondary to liver fibrosis in NAFLD, the most important prognostic parameter.

CONCLUSION

The 13C-methacetin breath test is a promising method of differentiating patients with NASH from patients with simple steatosis and could also predict significant fibrosis in these patients. This test could help in the functional evaluation and follow-up of patients with NAFLD, given the fact that an invasive liver biopsy is poorly regarded as a diagnostic and follow-up test for such a prevalent condition.

Conflicts of interest: Nothing to declare

REFERENCES

