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Role of ^{13}C methacetin breath test for non invasive staging of liver fibrosis in patients with chronic hepatitis C

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***Background & objectives*: The development and evaluation of non invasive tests to assess liver fibrosis have been an active field of research. The present study was carried out to evaluate the role of ^{13}C-methacetin breath test (^{13}C- MBT) as a non invasive tool for liver fibrosis staging in patients with chronic hepatitis C (CHC).**

***Methods*: ^{13}C-Methacetin breath test was performed in 115 patients with CHC histologically proven and in 55 healthy controls. All patients and controls underwent routine liver function tests. The CHC patients underwent histological assesment of liver by percutaneous liver biopsy. The correlation between the ^{13}C-methacetin breath test and liver biopsy was tested using Kendall's rank correlation coefficients. The overall validity was expressed as area under receiver operating characteristic curve (AUROC) with 95%CI.**

***Results*: Delta over baseline values (DOB) of CHC patients at 20 min were significantly reduced compared with control (16. 2 vs. 21. 06%, *P*<0.001). There were also significant differences between CHC patients and controls as regard the metabolization speed (dose /h at 20 min (17.80 vs 28.6, *P*<0.001) and metabolization capacity (cumulative recovery after 60 min (13.8 vs 20.4 *P*<0.001). The best ^{13}C-MBT parameter correlated with fibrosis was DOB at 20 min (r= - 0.596). The optimal cut-off for the diagnosis of advanced fibrosis (F≥3) was 15.2 per cent, with AUROC= 0.902, 95%CI: (0.851-0.938), a sensitivity of 82 per cent and a specificity of 80 per cent. DOB at 20 min predicted even better cirrhosis: AUROC = 0.932 95 per cent CI = 0.901-0.953, a sensitivity of 96 per cent and a specificity of 92 per cent.**

***Interpretation & conclusions*: Based on our findings the ^{13}C – methacetin breath test appears to be a promising tool to identify CHC patients with advanced fibrosis and to replace liver biopsy. Further studies need to be done to assess its potential to be used in regular clinical practice.**

Key words ^{13}C -methacetin breath test - hepatitis C - infrared spectrometry - liver fibrosis

It is generally accepted that the prognosis of chronic liver diseases is highly dependent on the extent of liver fibrosis. According to the recent guidelines[1] the treatment of chronic hepatitis C should be initiated in patients with significant fibrosis (F≥2). Liver biopsy is often used for the assessment of liver fibrosis but this is an invasive procedure with certain unavoidable risks and complications[2]. Therefore, the development of non invasive tests to assess hepatic inflammation and fibrosis has been an active area of research.

Several noninvasive methods have been proposed to stage liver fibrosis, including biochemical tests[3] and imaging techniques[4,5]. These static tests do not assess hepatic functional reserve[6,7]; this is possible only through dynamic tests, like the respiratory tests. In tests carbon (^{13}C) labelled substrates are used which are selectively metabolized to CO_2 in the liver. The measurement of the dynamic of $^{13}CO_2$ elimination in the exhaled air allows the assessment of the hepatic functional reserve[8].

The respiratory test that uses ^{13}C methacetin substrate is dedicated for the evaluation of hepatic microsomal function. ^{13}C methacetin is rapidly metabolized by hepatocytes through the cytochrome P 450 to acetaminophen and carbon dioxide ($^{13}CO_2$) through a single reaction of dealkylation. The quantitative determination of the carbon dioxide ($^{13}CO_2$) in the exhaled air can be achieved either by isotopic ratio mass spectrometry or non dispersive isotopic- selective infrared spectroscopy[8].

Only few studies using many patients were published until now. The aim of the present study was to evaluate the role of ^{13}C-methacetin breath as a non invasive tool for assessing the liver fibrosis staging in patients with chronic hepatitis C (CHC).

Material & Methods

Study design: Between February 2008 and March 2011, methacetin breath test (^{13}C MBT), was perfomed in 115 patients (55 males and 60 females) with CHC from a total 163 patients who underwent liver biopsy at the Medical Clinic II and Gastroenterology Department, University Hospital Bucharest, Bucharest, Romania, for treatment decision. Inclusion criteria were the presence of HCV RNA in serum and indicators of hepatic cytolysis [alanine aminotransferase (ALT) > 1.5 ×normal]. Patients with other forms of chronic liver disease, those with clinically advanced liver cirrhosis (Child –Pugh B and C) and with previous antiviral treatment were excluded from the study (11 patients). Patients taking P450 -cytochrome modulating capacity drugs, and those with lung and renal diseases (21 patients), were also excluded.

Hepatitis C virus (HCV) infection was defined by the presence of serum anti-HCV antibodies using the third generation ELISA (Ortho Diagnostics, High Wycombe, UK) HCV infection was confirmed by performing the COBAS TaqMan HCV test (Roche Molecular Systems, Inc., NJ, USA). This test is an *in vitro* amplification of the HCV nucleic acid, which uses the High Pure System Viral nucleic acid kit (Roche Diagnostics Corp, IN, USA) for manual preparation and the COBAS TaqMan 48 analyzer (Roche Diagnostics Corp, IN, USA) for automatic amplification and detection. The RNA titre was expressed in IU/ml. The detection limit was > 15 IU/ml with a positive rate of 95 per cent.

A total of 55 healthy medical persons, confirmed after annual medical check up (28 males and 27 females) served as controls. None had history of alcohol, medication intake or previous liver disease. Routine liver tests and ultrasonographic abdominal evaluation were normal.

All patients and healthy individuals provided written informed consent prior to enrollment in the study, and the study protocol was approved by the university ethics committee.

Biochemical evaluation of the liver: All patients underwent biochemical evaluation. Blood samples (20 ml) were obtained under fasting conditions and routine liver function tests [alanine transaminase (ALT) and aspartate transaminase (AST), total protein level, serum albumin and γ-globulins, γ-glutamyl transfpeptidase (GGT), total bilirubin, alkaline phosphatase, international normalized ratio (INR) were performed. All these were measured using Dade Behring reactants and the Dimension RXL analyzer (Dade Behring, FL, USA).

Ultrasonographic evaluation of the abdomen with Doppler measurements (portal flow volume and velocity) were performed in all patients and controls .

Histological assessment: All CHC patients underwent histological assessment by percutaneous liver biopsy. This was performed by using the Menghini technique with a 1.4 mm diameter needle (Hepafix; Braun, Germany). After biopsy, the liver samples were fixed in formalin, paraffin embedded, and stained with hematoxylin-eosin and Masson's trichrome. All biopsy

specimens were analyzed by an expert pathologist blinded to the patients' clinical results. The length of each liver biopsy was established in centimeters and the number of portal tracts was counted. Only liver fragments at least 1.5 cm in length and including 8 portal tracts were considered valuable for histological assessment. Using this criteria we excluded 16 patients.

Liver fibrosis and necroinflammatory activity were evaluated using the METAVIR scoring system[9]. Fibrosis was staged on a five-point scale according to this score: F0 indicated no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and a few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.

The AST-to-platelet ratio index (APRI) index: AST levels and platelet counts were measured with a Dimension RXL analyzer and a Hematology System analyzer (Beckman-Coulter, Inc., CA, USA). The APRI index was calculated in all patients as follows: AST (/Upper Limit of Normal) × 100/platelet count (109/l). The upper normal limit of AST was considered as 38 U/l.

^{13}C-methacetin breath test: According to the study protocol, all the patients and the control subjects underwent MBT. In CHC patients, MBT was performed within 1 month of the liver biopsy. After an overnight fast, each subject ingested 75 mg ^{13}C-methacetin (Wagner –Analysen-Technik,Bremen, Germany) dissolved in 200 ml water. Breath samples were collected at baseline and 10, 20, 30, 40, 50 and 60 min after ingestion of the substrate. The $^{13}CO_2$ isotope in the breath samples was analyzed by nondispersive isotope-selective infrared spectrometry (Wagner Analysen Technik GmbH Bremen/Germany). The results were expressed as peak value of breathing delta over baseline (DOB), the speed of metabolization (%^{13}C-dose/h) and the capacity of methacetin metabolization (% CUM) at different intervals of time. IRIS-data system (IRIS Lab version soft wave 3.01 ×) generated standardized graphs which allowed to compare patient's metabolisation parameters.

During the test period, all patients and healthy volunteers continued fasting and were at rest, to avoid any variability in CO_2 excretion due to the ingestion of food or physical activity.

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. The correlation between the non invasive tests and liver biopsy was tested using the Kendall's rank correlation coefficients. The overall validity was measured using area under the receiver operating characteristic curve (AUROC) with 95% CI to differentiate between CHC patients and healthy controls, and between the stages of fibrosis. Sensitivity, specificity, positive (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 $P < 0.05$.

Results

A total of 115 patients with chronic hepatitis C infection underwent ^{13}C –MBT and successful liver biopsy from a total of 163 patients. A group of 55 healthy volunteers who also performed ^{13}C -MBT served as control. The clinical and biochemical characteristics of the patients and healthy volunteers are presented in Table I.

Efficacy of ^{13}C-methacetin breath test in differentiating CHC patients from controls: Delta over baseline (DOB) values in controls showed the highest average at 20 minutes, (DOB 20: 22.06 ± 2.4%); this parameter was significantly lower in CHC patients compared with controls (DOB 20: 16.2 ± 4.2, P<0.001). The %dose/h at 20 min and CUM at 60 min were also significantly reduced in CHC patients compared with controls (17.8 vs 28.6, P<0.001, and 13.8 ± 4.7 vs 20.4 ± 2.6, P<0.001) respectively.

The ability of ^{13}C-MBT to differentiate liver disease patients from controls was demonstrated by ROC curves with areas under the curves as folows : DOB at 20min : 0.918 (95% CI = 0.872- 0.964, $P <$ 0.001), %dose/ h at 20 min : 0.914 (95% CI = 0.874-0.955, P< 0.001) and CUM60 0.942 (95% CI = 0.908-0.975, P< 0.001) (Figure).

Correlation of ^{13}C-MBT and fibrosis: ^{13}C-MBT was significantly correlated with METAVIR fibrosis score, (P<0.001). The Kendall's correlation coefficient was r=- 0.596 for DOB 20 and r= - 0.530 for CUM 60 . APRI index was calculated to compare this indicator of fibrosis with the ^{13}C-MBT results. Kendall's coefficient between APRI anf fibrosis was r=0.512, P<0.001 and inter test correlation (APRI/MBT) was r=-0.452 for DOB 20 and r=- 0.313 for CUM 60 (P<0.001).

Role of ^{13}C- MBT for non invasive staging of liver fibrosis: Patient distribution according to METAVIR fibrosis stage was : 10 patients without fibrosis F0 (8.69%), 23 patients with F1 (20%), 26 patients with

Table I. Characteristics of chronic hapatitis C patients and healthy controls

Characteristics	CHC patients (n=115)	Controls (n=55)
Sex (male/female)	55/60	28/27
Age (yr)	54.63 ±10.5 (31-68)	45.6 ±9.49 (25-65)
BMI (kg/m^2)	25.30 ±3.7	24.71 ± 4.01
AST (UI/l)	51.06 ± 30.35 (18 - 188)	12.8 ± 3.5 (18 -38)
ALT (UI/l)	65.82 ± 25.02 (20 - 147)	25.03 ± 6. 02 (22- 53)
INR	1.13 ± 0.17 (0.9 – 1.34)	1.10 ± 0.09 (0.9 – 1.13)
Bilirubin (mg/dl)	0.72 ± 0.41 (0.29-1.59)	0.69 ± 0.21 (0.29 – 1.08)
Albumin (g/dl)	3.9 ± 0.55	4.2 ± 0.63
Cholesterol (mg/dl)	192.36 ± 46. 58 (120 -311)	148.35± 68.29 (143 -257)
Triglycerides (mg/dl)	123.24 ± 73.33 (48 – 467)	116.96 ± 72.81 (42-287)

BMI, body mass index; AST, aspartate transaminase; ALT, alanine transaminase; INR, international normalized ratio

F2 (22.60%), 30 patients with F3 (26.08%) and 26 patients with cirrhosis, F4 (22.6%).

Table II presents the mean values of DOB 20 and CUM 60 for each stage of fibrosis. There were no significant differences between patients with early stages of fibrosis (F0 - F2) but MBT values were able to discriminate between advanced stages of fibrosis (F2/F3 and F3/F4). A high correlation was observed between decreasing methacetin metabolism and increasing stage of fibrosis.

To assess the sensitivity and specificity of MBT for detection of advanced fibrosis and cirrhosis ROC curve was used. For this purpose DOB 20 was identified as the best parameter for comparison between the groups (Table III). DOB at 20 min was a better predictor of F4 fibrosis (AUROC = 93.2%, 95% CI = 0.901-0.953) with a sensitivity of 96 per cent and a specificity of 92 per cent.

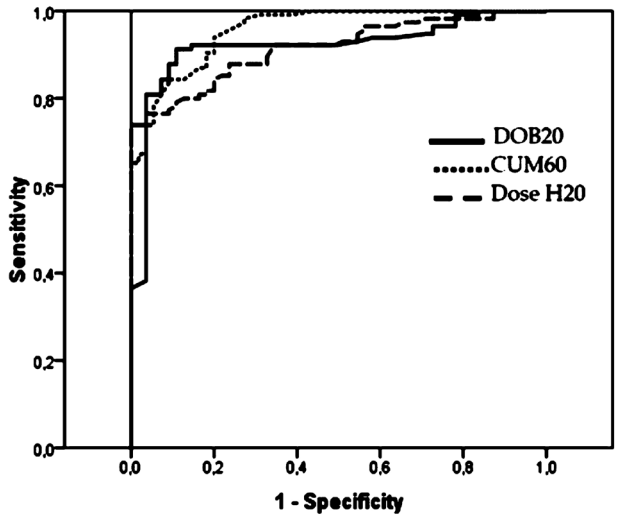

Fig. ROC analysis of the ^{13}C methacetin breath test to differentiate CHC patients from controls.

Discussion

Liver biopsy, considered the "gold standard" for the assessment of liver fibrosis, is not an optimal tool for patient management since it is highly invasive, expensive, and is very sensitive to sampling and analysis errors. This is the reason why the efforts to assess the stage of liver injury through non invasive methods are justified. All current non invasive methods for liver fibrosis staging have a diagnostic accuracy that does not exceed 85 per cent[10-13]. Thus, many patients still require a liver biopsy to establish the prognosis and the management of their liver disease. Navaneethan *et al*[14] have suggested that baseline severe fibrosis predicts a poor response to treatment.

Taking into account that the liver has a complex and high metabolic capacity, translated into detoxification, synthesis, metabolization, storage, an unique classical test may not reflect all these sides[15]. Moreover, each function may be differently affected in various stages of liver disease. To achive this, many studies have proposed several breath tests by using various labelled substrates: galactose[16], lidocaine[17], caffeine[18], aminopyrine[19], phenylalanine[20], methacetin[21]. Several studies have shown that specific cytochrome P450 IA2 proteins, which are expressed uniformly in the

Table II. DOB at 20 min and CUM at 60 min of the patients grouped according to the stage of fibrosis

Parameters	F0 (n=10)	F1 (n=23)	F2 (n=26)	F3 (n=30)	F4 (n=26)
DOB 20	18.4 ± 2.4	18.5 ± 3	17.7 ± 3**	14.6 ± 3.4***	8.8 ± 3.5
CUM 60	16.6 ± 1.3	16.7 ± 1.2	15.2 ± 2.5**	11.5 ± 3***	6.2 ± 2.4

Values are mean ± SD. *P* **<0.01 compared to F3, ***<0.001 compared to F4

Table III. Most discriminative cut-off values of DOB at 20 min according to fibrosis stage

Fibrosis	AUC (95% CI)	Cut-off %	Sn (%) (95% CI)	Sp(%) (95% CI)	LR+	LR-	PPV (%)	NPV (%)
F≥1	0.705 (0.612,0.785)	17.6	67 (56,76)	60 (26,88)	1.6	0.5	76	78
F≥2	0.793 (0.732,0.834)	16.9	79 (69,87)	75 (65,84)	3.25	0.28	76	78
F≥3	0.902 (0.851,0.938)	15.2	82 (70,91)	80 (72,87)	4.5	0.23	68	90
F=4	0.932 (0.901,0.953)	13	96 (80,100)	92 (87,96)	12.6	0.07	69	99

Sn, sensitivity; Sp, specificity; PPV & NPV, positive and negative predictive value; LR + & LR-, positive and negative likelihood ratio; AUC, area under curve

acinus, are reduced in different stages of chronic liver diseases[22-24]. Previous data suggest that ^{13}C-MBT is reliable in predicting the severity of cirrhosis without any evidence of toxicity related to the small doses of substrate used in contrast to other breath tests[25,26].

Giannini *et al*[19]demonstrated that the ^{13}C-aminopyrine breath test was able to differentiate chronic asymptomatic hepatitis from patients with cirrhosis, but the aminopyrine had the disadvantage of a slow clearance rate and the risk of serious side effects such as agranulocytosis. Other studies using the ^{13}C-galactose breath test in patients with chronic hepatitis C showed a good correlation with the severity of liver fibrosis[16,20].

Zhang *et al*[20] showed that the ^{13}C-phenylalanine test represented a good index of hepatic metabolic capacity with additional benefits reflected in its safety. Their results showed that the ^{13}C-phenylalanine breath test discriminated liver function not only between healthy subjects and liver disease patients, but also between different scores of cirrhosis patients. Using two substrates, phenylalanine and methacetin, it was shown that methacetin had a better diagnostic capacity due to its superior pharmacokinetics[23]. Also, the ^{13}C -metacethin test had a good correlation with the Child-Pugh score in cirrhotic patients and was used as a follow up tool for chronic liver disease[27].

In this context, the aim of our study was to evaluate the role of ^{13}C-methacetin breath test as a non invasive tool for liver fibrosis staging in patients with chronic hepatitis C. Our results showed that there were significant differences in the methacetin metabolization not only between controls and CHC patients, but also between patients with different stages of fibrosis.

Early and rapid detection of advanced fibrosis using this non invasive procedure is essential since patients with advanced fibrosis are at a higher risk to develop complications, such as portal hypertension or hepatocellular carcinoma, and consequently need specific follow up.

In another study DOB at 15 min was lower in patients with chronic hepatitis C infection versus controls and also it had high sensitivity (95 %) and specificity (96%) in identifying patients with cirrhosis[27]. In cirrhotic patients, the ^{13}C-MBT attained distinctly higher sensitivity and specificity compared with other biomarkers of cirrhosis (the AST/ ALT and APRI index). The authors reported an overlap of ^{13}C-MBT results between controls and patients with early stages of fibrosis. Regarding the role of ^{13}C-MBT in

the early diagnosis of fibrosis, our study indicated that there were no significant differences between patients with early stages of fibrosis (F0 - F2) according to methacetin metabolization. Our study confirmed the findings of Goetze *et al*[28]. regarding the ^{13}C-MBT's ability to differentiate between patients with high grade and low grade of fibrosis.

Another study has demonstrated that breath tests may usefully contribute to non invasive characterization of patients with non-alcoholic fatty liver disease[29]. Lock *et al*[30] suggested for correct interpretation of breath test results to take into account the bicarbonate kinetics, due to partial transportation of CO_2 as plasma bicarbonate. However, in our study patients with respiratory and renal disturbances were excluded.

The major advantage of ^{13}C-MBT compared with liver biopsy (1/50,000 of the total liver mass) is that it is painless, rapid, free of complications, well tolerated and is more representative for the entire liver parenchyma[31]. Compared with other non invasive tests, it is easily performed and gives real time information about liver function.

Because there is no consensus about normal values of the ^{13}C-MBT parameters in the literature, our healthy control results served as reference. This could be a limitation of our study. Another limitation was that we did not use a validated non ivasive test –FibroScan- for comparison.

In conclusion, the ^{13}C –methacetin breath test appears to be a reliable tool to identify CHC patients with advanced fibrosis, at risk for liver complications. Offering complementary information about liver function, ^{13}C-MBT has a potential to be used in the clinical practice.

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