^{13}C-methacetin breath test in the evaluation of disease severity in patients with liver cirrhosis

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Aims. The non-invasive ^{13}C-methacetin (^{13}C-MBT) breath test has been proposed as a measure of metabolic liver function that improves the diagnostic efficacy of serologic and biochemical tests in assessing hepatic functional capacity and liver disease severity, The goal of this study was to establish the clinical utility of this test in quantifying hepatic metabolic function in patients with liver cirrhosis of varying severity and to compare ^{13}C-MBT measurements with the AST/ALT ratio, APRI score, and other routine liver tests.

Methods. Routine liver function tests including serum bilirubin, aspartate aminotransferase activity (AST), alanine aminotransferase (ALT), AST/ALT ratio, the APRI score, the percentage of dose rate (PDR) and cumulative percentage of dose rate (CPDR) of the ^{13}C-MBT were evaluated in 52 cirrhotic patients of alcohol etiology (Child-Pugh A/B/C 10/28/14) and 37 healthy controls.

Results. The ^{13}C-MBT differed significantly between healthy controls and cirrhotic patients at all time intervals measured. It also proved the ability to differentiate patients with liver cirrhosis based on severity of hepatic impairment corresponding to the Child-Pugh classification A vs. B vs. C. The ROC curve analysis suggested that the best prediction is provided by time intervals between the 10th - 20th or 10th - 40th minute of PDR.

Conclusions. The ^{13}C-MBT offers a reliable means for quantification of hepatic metabolic function over the complete range of functional liver impairment. It is non-invasive, easy to perform and completely safe.

Key words: ^{13}C-methacetin breath test, cirrhosis, Child-Pugh classification, non-dispersive infrared spectroscopy, aldehyde dehydrogenase, alcohol dehydrogenase, cytochrome P450, catalase

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INTRODUCTION

Quantification of liver function, usually metabolic, is carried out to assess liver function reserve, e.g. prior to hepatic resection, to establish a prognosis and/or assess the effect of treatment.

Standard serologic and biochemical tests do not accurately assess hepatic functional capacity nor do they detect changes in hepatic disease severity[1-3]. To improve the diagnostic efficacy of these tests, several quantitative approaches have been proposed to measure metabolic liver function. Currently, breath tests using carbon isotope ^{13}C are preferentially used[4,5]. The ^{13}C is biologically identical to ^{12}C, it is non-toxic and non-radioactive. For this reason, it can be safely used for diagnostic purposes. ^{13}C can be incorporated into organic substances the metabolic breakdown of which is assessed by measuring the amount of $^{13}CO_2$ in exhaled air[6]. A substance widely used for breath tests is ^{13}C-labeled methacetin. The ^{13}C-labeled carbon dioxide is exhaled and the volume exhaled is a measure of microsomal hepatic function provided by the activity of cytochrome P450. Measuring the $^{13}CO_2/^{12}CO_2$ ratio in exhaled air enables quantification of the microsomal function by means of two variables: kinetics, i.e. percentage of ^{13}C dose being actually exhaled and capacity, i.e. cumulative dose of ^{13}C exhaled to the time point studied.

^{13}C-methacetin is metabolized in the endoplasmic reticulum of hepatocytes by means of cytochrome P450, exclusively by isoenzyme CYP1A2, by demethylation and oxidation to acetaminophen and $^{13}CO_2$ (ref.[7-10]). CYP1A2 also contributes to ethanol metabolism in the microsomes[8], thus the ^{13}C-methacetin breath test (^{13}C-MBT) provides the most valuable results in cases of alcoholic liver disease[9]. Since the Czech Republic is reported to have the highest adult per capita alcohol consumption in the European Union (16.61 liters in 2009) and since 50% of all cases of liver cirrhosis in the Czech Republic have an alcoholic etiology[11,12], we decided to concentrate on patients with alcoholic liver disease.

The ^{13}C-MBT could not distinguish between controls and patients in early stages of fibrosis nor could they indicate the extent of inflammation or the degree of fibrosis[13]. However, few studies show good correlation of the ^{13}C-methacetin breath test with the severity of liver cirrhosis according to the Child-Pugh score[6,14,15].

Thus main goals of our study were (a) to establish the ^{13}C-MBT sensitivity and specificity for the evaluation of liver function in healthy controls and patients with liver cirrhosis of varying severity based on the Child-Pugh score. Emphasis was placed on exploration, whether the ^{13}C-MBT can also predict early stages of clinically nonsymptomatic cirrhosis (Child-Pugh A)) and on comparing healthy controls with all cirrhotic patients together, (b) to determine the best discriminating time interval of the ^{13}C-MBT for all pairs of groups of patients, and (c) to compare the discriminating power of conventional liver tests, APRI score, AST/ALT ratio, and serum bilirubin levels with the diagnostic accuracy of ^{13}C-MBT.

MATERIALS AND METHODS

Patients

Altogether, 52 cirrhotic patients and 37 healthy controls participated in the study. The liver cirrhosis group consisted of 52 patients (mean age 59.23 ± 9.87, range 36 - 80; Female/Male ratio (F/M) 26/26, mean value of body mass index (BMI) was 25.25 ± 6.08, the cirrhosis was of alcoholic etiology only). The diagnosis of liver cirrhosis was confirmed by ultrasound examination, liver function test and/or biopsy. All patients were classified by the Child-Pugh classification, comprising ascites, encephalopathy, serum albumin, bilirubin levels and prothrombin time. The Child-Pugh classification[16,17], which represents a concerted evaluation of clinical criteria and laboratory data, still remains the most widely accepted prognostic measure of the severity of liver diseases. The functional Child-Pugh classification was as follows: 10 patients were in class A (mean age 65.10 ± 8.28; BMI 23.59 ± 8.24; F/M 7/3; scores 5-6), 28 in class B (mean age 59.11 ± 9.22; BMI 26.44 ± 5.56; F/M 14/14; scores 7-9) and 14 in class C (mean age 55.29 ± 10.72; BMI 24.04 ± 5.21; F/M 5/9; scores 10-15). The control group consisted of 37 healthy volunteers (mean age 58.81 ± 12.6, range 38 - 90 years, female/male ratio 15/22, BMI 27.27 ± 4.11). They were screened through a medical history, physical examination, liver ultrasound and routine liver function tests. All healthy volunteers had blood tests within normal limits. None had a history of active or previous liver disease or alcohol or drug abuse. All subjects participating in the study were examined at the Department of Medicine I, 3rd Faculty of Medicine, Charles University in Prague between 2000 - 2011. Written informed consent was obtained from all patients and healthy individuals, and the study was approved by the local Ethics Committee of the Third Faculty of Medicine, Charles University in Prague.

^{13}C-Methacetin breath test

After an overnight fasting all patients were administered ^{13}C-methacetin orally at a dose of 75 mg. During the breath test, subjects were required to remain at rest, without eating, drinking or smoking. Breath samples were collected before and 10, 20, 30, 40, 50, 60, 80, 100 and 120 minutes after administration. The total amount of CO_2 and its $^{13}C/^{12}C$ ratio were analyzed using isotope-selective non-dispersive infrared spectroscopy (IRIS II, Wagner Analysen Technik, Bremen, Germany). The description of the device and the physical-chemical principle of its function were previously described[18].

The measuring instrument produces a value $\delta(t_i)$ (delta over base line DOB = $\delta_t - \delta_0$ [‰], where δ_0 is a delta value before taking the test substrate (base line) and δ_t is a delta value at time *t* after taking the substrate). The instantaneous substrate-origin-^{13}C content – percentage dose recovery (PDR) of $^{13}CO_2$ [%/h] and cumulated substrate-origin-^{13}C content – cumulative percentage dose recovery (CPDR) of ^{13}C recovered over the testing period [%] are calculated from $\delta(t_i)$.

The PDR is calculated using the formula[19]:

$$PDR = \frac{\frac{\left({}^{13}\delta_t - {}^{13}\delta_0\right) + \left({}^{13}\delta_{t+1} - {}^{13}\delta_0\right)}{2} \times \left(t_{t+1} - t\right) \times R_{PDB} \times CO_2 \times 10^{-3}}{\frac{mg\ substrate}{mol\ weight} \times \frac{P \times n}{100}} \times 100\%,$$

where $^{13}\delta = 1000 \times [(R_{meas}/R_{PDB}) - 1]$, R_{meas} denotes the $^{13}CO_2/^{12}CO_2$ isotope ratio in the sample at time $t \geq 0$ and $R_{PDB} = {}^{13}CO_2/{}^{12}CO_2$ in PDB (natural abundance standard for carbon is calcium carbonate of a fossil Belemnitella of the Pee-Dee-formation in South Carolina, R_{PDB}= 0.01123686); P is the atom % excess; n is the number of ^{13}C-labelled atom; δ_t, δ_{t+1}, δ_0 are enrichments at times t, t_{t+1} and predose respectively. CO_2 is the CO_2 production rate = 300 mmol/h m^2 of BSA, where body surface area $BSA = 0.024265 \times \text{Weight}^{0.5378} \times \text{Height}^{0.3964}$ was calculated using the Haycock formula[20]. A cumulative PDR can be obtained by summation of the single PDR value for each time interval and represents the area under the curve obtained during the duration of the test[19].

On the day of the study, liver function tests including serum bilirubin (BILI), the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined. The ultrasonographic evaluation of the abdomen and echocardiographic studies were also performed on the day of the breath test. The AST/ALT ratio and APRI score was calculated for all patients and healthy controls.

Statistical analysis

Statistical analysis of normally distributed variables (routine liver tests (ALT, AST, and bilirubin) and the ^{13}C-methacetin breath test) was carried out using the independent sample t-test to compare all cirrhosis patients together, i.e. Child-Pugh A+B+C (Cirrhosis) vs. Controls and using one-way analysis of variance ANOVA to compare all groups of patients, i.e. Child-Pugh A (ChPA) vs. Child-Pugh B (ChPB) vs. Child-Pugh C (ChPC) vs. Controls. Differences among groups were compared using the multiple comparison post-hoc Bonferroni method. For comparison of skewed variables (AST/ALT ratio and APRI score) the non-parametric Mann-Whitney U test and Kruskal-Wallis ANOVA with multiple comparisons of mean ranks were used.

Receiver operating characteristic curve (ROC) analysis was performed to evaluate the best discriminating time

interval of ^{13}C-methacetin breath test and to compare the discriminating power of simple liver biochemical indicators (ALT, AST, and bilirubin), AST/ALT ratio, APRI score and the ^{13}C-methacetin breath test. Lastly, in order to determine whether there was a statistically significant difference in diagnostic power between the best routine liver tests and the best time interval of ^{13}C-methacetin breath test, the *Z*-statistics defined by Hanley and McNeil was calculated[21]. Statsoft's STATISTICA version 9 was used for statistical analysis. A *P*-value equal to or less than 0.05 was considered to be statistically significant.

RESULTS

Clinical and laboratory data are given as means and standard deviations, for AST/ALT ratio and APRI score the median and range are added (Table 1). Mean values of kinetics (%^{13}C-dose/h) and capacity (%^{13}C-cumulative dose) for all groups of patients are displayed in Fig. 1 and 2.

Comparison of healthy controls with all cirrhotic patients together

The differences in all normally distributed observed variables between the cirrhotic patients and healthy controls were compared using the independent sample t-test. Statistically significant differences were found in all variables except ALT: in BILI (P=0.0016), in AST and in all measured values of PDR and CPDR in all time intervals of ^{13}C-MBT (P<0.001). Using Mann-Whitney U test the remarkably statistically significant differences (P<0.0001) were found for both variables, AST/ALT ratio and APRI score.

Comparison of healthy controls with patients with liver cirrhosis of various severity based on the Child-Pugh score

One-way analysis of variance ANOVA was conducted to find statistically significant differences among all four groups of patients for (i) all laboratory biochemical results, and (ii) breath tests. Non-parametric Kruskal-Wallis ANOVA was used for (iii) the AST/ALT ratio, and (iv) the APRI score.

The ANOVA test was statistically significant, again, for all variables except ALT. However, using the multiple comparison Bonferroni test, statistically significant differences were only found in the following cases: none of the single biochemical tests could distinguish among ChPA, ChPB and ChPC groups of patients, except bilirubin, when there was a significant difference between groups ChPA vs. ChPC (P=0.048). All routine biochemical tests, except ALT, were found to differentiate between Controls and ChPB: BILI (P=0.018), AST (P<0.001), and between Controls and ChPC: BILI (P=0.003), AST (P=0.002).

The Kruskal-Wallis ANOVA was statistically significant for both variables, for AST/ALT ratio and APRI score. The multiple comparisons of mean ranks found statistically significant differences in the following cases: for AST/ALT ratio: Controls vs. ChPB and Controls vs. ChPC (P<0.001) and for APRI score: Controls vs. ChPB and Controls vs. ChPC (P<0.0001).

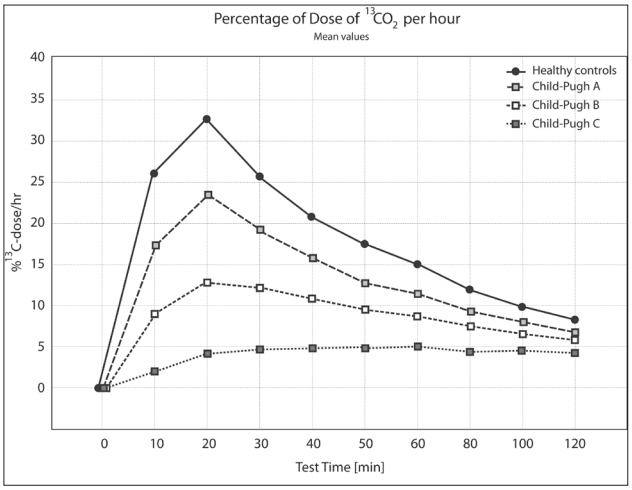

Fig. 1. Percentage of dose of administered ^{13}C recovered per hour in four groups of patients (mean values) for all time test intervals.
Group 1: Child-Pugh A (n = 10), Group 2: Child-Pugh B (n = 28), Group 3: Child-Pugh C (n = 14) and Group 4: healthy controls (n = 37).

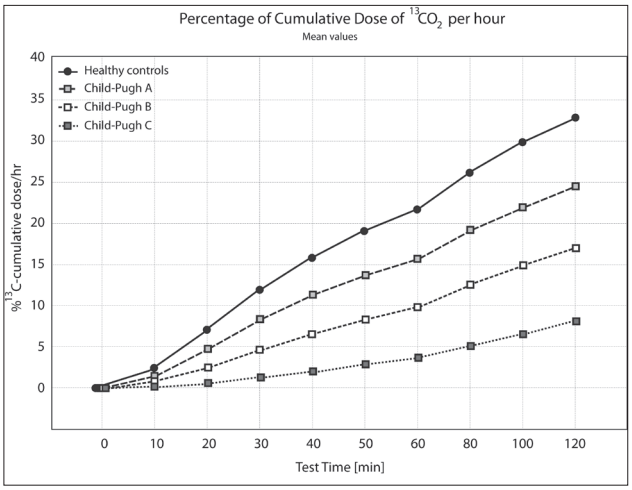

Fig. 2. Percentage of cumulative dose of administered ^{13}C recovered per hour in four groups of patients (mean values) for all time test intervals.
Group 1: Child-Pugh A (n = 10), Group 2: Child-Pugh B (n = 28), Group 3: Child-Pugh C (n = 14) and Group 4: healthy controls (n = 37).

No biochemical test alone nor AST/ALT ratio nor APRI score is able to differentiate between Controls and ChPA.

In the ^{13}C-methacetin breath test, statistically significant differences between ChPA and ChPB groups were found only in the 20th minute of PDR (P=0.042) and in the 30th minute of CPDR (P=0.049). Statistically remarkably significant differences between groups ChPA and ChPC were found in the following cases: for PDR in 10th, 20th, 30th, 40th, 50th, 60th, 80th and 100th minute (in all cases P<0.001); for CPDR statistically significant differences

Table 1. Main characteristic for all patients groups: selected biochemical tests and values of dose and cumulative dose for all time intervals (mean value and standard deviation) and p-values of t-test (or Mann-Whitney U test) and ANOVA (or Kruskal-Wallis ANOVA).

	Child-Pugh A	Child-Pugh B	Child-Pugh C	Cirrhosis	Controls	†Controls vs. ChPA	†Controls vs. ChPB	†Controls vs. ChPC	†ChPA vs. ChPB	†ChPA vs. ChPC	†ChPB vs. ChPC	††Controls vs. Cirrhosis
			Mean ± SD						*P*-values			
Biochemical tests												
BILI (μmol/L)	13.88 ± 5.37	58.93 ± 10.20	81.76 ± 9.24	56.41 ± 8.26	12.07 ± 2.38	1.000	**0.018**	**0.003**	0.276	**0.048**	1.000	**0.002**
ALT (μkat/L)	0.60 ± 0.37	0.78 ± 0.56	0.87 ± 0.76	0.77 ± 0.59	0.59 ± 0.32	1.000	0.891	0.479	1.000	1.000	1.000	0.104
AST (μkat/L)	0.75 ± 0.28	1.40 ± 1.21	1.52 ± 1.10	1.31 ± 1.09	0.53 ± 0.21	1.000	0.0005	**0.0017**	0.221	0.161	1.000	**<0.001**
AST/ALT ratio												
Mean ± SD	1.38 ± 0.49	1.94 ± 1.53	1.92 ± 0.79	1.83 ± 1.22	1.07 ± 0.67							
Median	1.46	1.53	1.67	1.55	0.86	0.308	**<0.0001***	**<0.001***	1.000	0.696	1.000	**<0.0001***
Min – Max	0.67 – 2.03	0.40 – 8.57	1.09 – 4.05	0.40 – 8.57	0.39 – 4.17							
APRI score												
Mean ± SD	0.85 ± 0.55	1.88 ± 1.61	1.99 ± 2.06	1.74 ± 1.78	0.35 ± 0.23							
Median	0.66	1.36	1.40	1.18	0.31	0.086	**<0.0001***	**<0.00001***	1.000	0.636	1.000	**<0.0001***
Min – Max	0.20 – 1.67	0.40 – 6.43	0.20 – 7.62	0.20 – 7.62	0.09 – 1.24							
^{13}C-MBT parameters												
PDR10 (%/h)	17.22 ± 11.43	9.15 ± 11.17	1.91 ± 3.50	8.75 ± 10.92	25.86 ± 9.30	**0.049**	**<0.00001**	**<0.00001**	0.145	**0.0012**	0.137	**<0.00001**
PDR20 (%/h)	23.27 ± 10.68	12.93 ± 12.54	4.04 ± 5.41	12.53 ± 12.38	32.48 ± 9.31	0.059	**<0.00001**	**<0.00001**	**0.042**	**<0.001**	0.053	**<0.00001**
PDR30 (%/h)	19.09 ± 8.62	12.22 ± 10.62	4.65 ± 5.11	11.50 ± 10.19	25.57 ± 6.16	0.166	**<0.00001**	**<0.00001**	0.141	**<0.001**	**0.032**	**<0.00001**
PDR40 (%/h)	15.67 ± 6.65	10.87 ± 8.32	4.80 ± 4.47	10.16 ± 7.98	20.70 ± 5.12	0.259	**<0.00001**	**<0.00001**	0.244	**0.0004**	**0.024**	**<0.00001**
PDR50 (%/h)	12.70 ± 5.26	9.65 ± 6.67	4.81 ± 4.17	8.93 ± 6.37	17.44 ± 4.62	0.126	**<0.00001**	**<0.00001**	0.563	**0.0012**	**0.019**	**<0.00001**
PDR60 (%/h)	11.38 ± 4.29	8.78 ± 5.52	5.01 ± 3.63	8.27 ± 5.26	14.89 ± 3.31	0.219	**<0.00001**	**<0.00001**	0.520	**0.0018**	**0.035**	**<0.00001**
PDR80 (%/h)	9.29 ± 3.27	7.56 ± 4.34	4.37 ± 3.41	7.03 ± 4.23	11.88 ± 2.09	0.253	**<0.00001**	**<0.00001**	0.904	**0.003**	**0.022**	**<0.00001**
PDR100 (%/h)	7.90 ± 3.01	6.62 ± 3.43	4.49 ± 3.26	6.29 ± 3.46	9.75 ± 1.91	0.551	**0.0004**	**<0.00001**	1.000	**0.026**	0.137	**<0.00001**
PDR120 (%/h)	6.74 ± 2.11	5.91 ± 2.93	4.26 ± 2.81	5.62 ± 2.85	8.27 ± 1.50	0.514	**0.0013**	**<0.00001**	1.000	0.070	0.197	**<0.00001**
CPDR10 (%)	1.43 ± 0.95	0.76 ± 0.93	0.16 ± 0.29	0.73 ± 0.91	2.15 ± 0.77	**0.049**	**<0.00001**	**<0.00001**	0.145	**0.0012**	0.137	**<0.00001**
CPDR20 (%)	4.81 ± 2.63	2.60 ± 2.81	0.65 ± 1.03	2.50 ± 2.77	7.02 ± 2.17	**0.035**	**<0.00001**	**<0.00001**	0.068	**0.0002**	0.071	**<0.00001**
CPDR30 (%)	8.34 ± 4.03	4.70 ± 4.63	1.38 ± 1.90	4.50 ± 4.55	11.85 ± 3.17	**0.037**	**<0.00001**	**<0.00001**	**0.049**	**<0.001**	**0.041**	**<0.00001**
CPDR40 (%)	11.24 ± 5.18	6.62 ± 6.13	2.17 ± 2.69	6.31 ± 6.00	15.71 ± 3.72	**0.045**	**<0.00001**	**<0.00001**	0.056	**<0.001**	**0.029**	**<0.00001**
CPDR50 (%)	13.60 ± 6.11	8.33 ± 7.33	2.97 ± 3.40	7.90 ± 7.15	18.89 ± 4.12	0.052	**<0.00001**	**<0.00001**	0.070	**<0.0001**	**0.024**	**<0.00001**
CPDR60 (%)	15.61 ± 6.87	9.87 ± 8.31	3.79 ± 4.03	9.33 ± 8.08	21.58 ± 4.48	0.056	**<0.00001**	**<0.00001**	0.086	**<0.0001**	**0.022**	**<0.00001**
CPDR80 (%)	19.05 ± 8.04	12.59 ± 9.88	5.35 ± 5.13	11.88 ± 9.58	26.04 ± 5.09	0.065	**<0.00001**	**<0.00001**	0.114	**<0.0001**	**0.020**	**<0.00001**
CPDR100 (%)	21.92 ± 8.98	14.95 ± 11.08	6.83 ± 6.14	14.11 ± 10.76	29.65 ± 5.56	0.073	**<0.00001**	**<0.00001**	0.143	**0.0002**	**0.020**	**<0.00001**
CPDR120 (%)	24.36 ± 9.72	17.04 ± 12.04	8.28 ± 7.04	16.09 ± 11.69	32.65 ± 5.94	0.082	**<0.00001**	**<0.00001**	0.173	**0.0002**	**0.022**	**<0.00001**

† *P*-values of multiple comparison Bonferroni test for one-way analysis of variance ANOVA or p-values of multiple comparisons of mean ranks for Kruskal-Wallis ANOVA (*) among four groups of patients: Child-Pugh A (n = 10) vs. Child-Pugh B (n = 28) vs. Child-Pugh C (n = 14) vs. Controls (n =37)

†† *P*-values of t-test or non-parametric Mann-Whitney U test (*) for comparison between all cirrhotic patients together (n = 52) and Controls (n = 37)

Statistically significant values are marked in bold

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Table 2. Area under the receiver operating characteristic (ROC) curve for biochemical tests and ^{13}C-methacetin breath test among four groups of patients: Child-Pugh A (n = 10) vs. Child-Pugh B (n = 28) vs. Child-Pugh C (n = 14) vs. Controls (n = 37).

	Controls vs. Child-Pugh A		Controls vs. Child-Pugh B		Controls vs. Child-Pugh C		Child-Pugh A vs. Child-Pugh B		Child-Pugh A vs. Child-Pugh C		Child-Pugh B vs. Child-Pugh C		Controls vs. Cirrhosis	
	AUC	SE	AUC	SE	AUC	SE	AUC	SE	AUC	SE	AUC	SE	AUC	SE
1st part – Biochemical tests														
BILI (μmol/L)	0.660	0.104	0.876†	0.047	0.994	0.012	0.771	0.070	0.971	0.000	0.827	0.074	0.867	0.038
ALT (μkat/L)	0.540	0.102	0.587	0.072	0.628	0.091	0.605	0.101	0.718	0.105	0.467	0.096	0.573	0.062
AST (μkat/L)	0.741	0.094	0.802	0.058	0.927	0.050	0.666	0.095	0.789	0.093	0.566	0.096	0.829	0.043
AST/ALT	0.661	0.103	0.759	0.062	0.837	0.071	0.632	0.099	0.693	0.108	0.584	0.096	0.761	0.050
APRI	0.762*	0.093	0.834	0.055	0.960	0.052	0.657	0.095	0.754	0.093	0.551	0.096	0.864	0.041
2nd part – ^{13}C-MBT parameters														
PDR10 (%/h)	0.722	0.083	0.883	0.042	0.992	0.011	0.750	0.098	1.000	0.039	0.829	0.063	0.881	0.039
PDR20 (%/h)	0.735	0.081	0.878	0.043	0.992	0.011	0.818	0.095	0.964	0.042	0.814	0.071	0.881	0.039
PDR30 (%/h)	0.722	0.083	0.844	0.048	0.981	0.017	0.754	0.098	0.957	0.048	0.781	0.071	0.857	0.043
PDR40 (%/h)	0.716	0.084	0.823	0.051	0.973	0.021	0.704	0.103	0.943	0.055	0.765	0.073	0.843	0.045
PDR50 (%/h)	0.727	0.082	0.813	0.053	0.958	0.026	0.639	0.107	0.914	0.067	0.750	0.076	0.853	0.046
PDR60 (%/h)	0.716	0.084	0.804	0.054	0.958	0.026	0.636	0.107	0.886	0.076	0.732	0.073	0.828	0.047
PDR80 (%/h)	0.765	0.080	0.798	0.054	0.944	0.031	0.646	0.107	0.843	0.088	0.735	0.078	0.826	0.047
PDR100 (%/h)	0.673	0.090	0.772	0.057	0.917	0.038	0.618	0.108	0.786	0.100	0.684	0.084	0.792	0.050
PDR120 (%/h)	0.722	0.083	0.761	0.059	0.894	0.044	0.614	0.108	0.786	0.100	0.666	0.086	0.789	0.051
CPDR10 (%)	0.722	0.083	0.883	0.042	0.992	0.011	0.750	0.098	1.000	0.039	0.829	0.063	0.881	0.039
CPDR20 (%)	0.724	0.082	0.875	0.043	0.992	0.011	0.761	0.097	1.000	0.039	0.814	0.065	0.878	0.040
CPDR30 (%)	0.708	0.085	0.870	0.044	0.992	0.011	0.757	0.097	0.964	0.043	0.796	0.069	0.872	0.041
CPDR40 (%)	0.711	0.084	0.870	0.044	0.996	0.009	0.757	0.097	0.964	0.043	0.786	0.070	0.873	0.041
CPDR50 (%)	0.703	0.086	0.867	0.045	0.996	0.009	0.754	0.098	0.964	0.043	0.796	0.069	0.870	0.041
CPDR60 (%)	0.711	0.084	0.864	0.048	0.990	0.012	0.743	0.099	0.964	0.043	0.786	0.070	0.869	0.041
CPDR80 (%)	0.708	0.085	0.858	0.046	0.986	0.014	0.736	0.100	0.964	0.043	0.776	0.072	0.864	0.042
CPDR100 (%)	0.700	0.086	0.851	0.047	0.985	0.015	0.718	0.102	0.950	0.051	0.768	0.073	0.858	0.043
CPDR120 (%)	0.700	0.086	0.847	0.048	0.979	0.018	0.707	0.103	0.936	0.058	0.765	0.073	0.854	0.043

* Black – the best discriminator for the given two groups of patients
† Light gray – the second best discriminators
SE – standard error of AUC

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Table 3. The comparison of the classification accuracy and discriminating power between the best routine liver test and the best time interval of ^{13}C-methacetin breath test using ROC analysis.

Group vs. Group	The best discriminator from routine liver tests						The best discriminator from ^{13}C-methacetin breath test						Comparison of ROC curves†
	Test	AUC	Cutoff	Sensitivity	Specificity	Accuracy*	Test	AUC	Cutoff	Sensitivity	Specificity	Accuracy*	
Controls vs. ChPA	AST[a] (μkat/L)	0.741	> 0.68	70.0	88.9	FAIR							0.776
	AST/ALT	0.661	> 1.40	60.0	83.3	POOR	PDR80 (%/h)	0.765	≤ 11.71	90.0	76.8	FAIR	0.447
	APRI	0.762	> 0.86	70.0	83.3	FAIR	PDR20 (%/h)‡	0,735	≤ 27.39	70.0	67.9	FAIR	0.798
Controls vs. ChPB	BILI[a](μmol/L)	0.876	> 21.6	67.9	94.3	GOOD							0.801
	AST/ALT	0.759	> 1.17	85.7	52.8	FAIR	PDR10 (%/h)	0.883	≤ 16.56	85.7	86.5	GOOD	0.080
	APRI	0.834	> 0.66	67.9	94.4	GOOD							0.402
Controls vs. ChPC	BILI[a](μmol/L)	0.994	> 25.4	92.9	100.0	EXCELLENT							0.895
	AST/ALT	0.837	> 1.29	85.7	63.9	GOOD	CPDR50 (%)	0.996	≤ 4.50	100.0	97.1	EXCELLENT	**0.024**
	APRI	0.960	> 0.90	92.9	88.9	EXCELLENT							0.372
ChPA vs. ChPB	BILI[a](μmol/L)	0.771	> 20.6	67.9	80.0	FAIR							0.647
	AST/ALT	0.632	> 1.48	57.1	60.0	POOR	PDR20 (%/h)‡	0.818	≤ 16.80	67.9	100.0	GOOD	0.139
	APRI	0.657	> 1.67	42.9	100.0	POOR							0.396
ChPA vs. ChPC	BILI[a](μmol/L)	0.971	> 20.6	92.9	100.0	EXCELLENT							0.463
	AST/ALT	0.693	> 1.35	71.4	40.0	POOR	PDR10 (%/h)	1.000	≤ 2.65	100.0	100.0	EXCELLENT	**0.005**
	APRI	0.754	> 0.63	92.9	50.0	FAIR							**0.034**
ChPB vs. ChPC	BILI[a](μmol/L)	0.827	> 59.6	71.4	89.3	GOOD							0.974
	AST/ALT	0.584	> 1.54	64.3	53.6	FAIL	PDR10 (%/h)	0.829	≤ 1.87	85.7	85.7	GOOD	**0.017**
	APRI	0.551	> 0.86	71.4	42.9	FAIL	PDR20 (%/h)‡	0.814	≤ 6.08	79.6	82.1	GOOD	**0.007**
Controls vs. Cirrhosis	BILI[a](μmol/L)	0.867	> 14.6	76.9	82.9	GOOD							0.665
	AST/ALT	0.761	> 1.17	84.6	52.8	FAIR	PDR20 (%/h)	0.881	≤ 18.93	75.0	94.6	GOOD	**0.034**
	APRI	0.864	> 0.90	78.8	86.1	GOOD							0.728

* Classification of the accuracy of a diagnostic test in the traditional academic point system [Tape]
† *P*-values of Z-statistics defined by Hanley and McNeil (statistically significant differences marked in bold)
[a] Best biochemical discriminator
‡ The best expression of the whole ^{13}C-MBT

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were found in all time intervals (P<0.001). Finally, statistically significant differences between ChPB and ChPC groups were found in the 30th, 40th, 50th, 60th, 80th, 100th and 120th minute for both PDR and CPDR (in all cases P<0.001). Comparing healthy controls with Child-Pugh groups of patients the following results were found: in comparisons between Controls vs. ChPB and Controls vs. ChPC statistically significant differences were found in all time intervals of PDR and CPDR (P<0.001 in all time intervals). The statistically significant differences were also found between Controls and ChPA, but only in the 10th minute of PDR (P=0.049) and in the 10th, 20th, 30th and 40th minute of CPDR (P<0.05).

Estimation of the best discriminating time interval of ^{13}C-MBT

The ROC analysis was used (i) to find the best discriminating time interval for the ^{13}C-methacetin breath test between all pairs of groups of patients (the greatest area under the ROC curve) details are shown in Table 2 and (ii) to compare the discriminating power and classification accuracy between the best routine liver function tests, AST/ALT ratio and the APRI score on one hand and the best time interval for the ^{13}C-methacetin breath test on the other hand and to determine the best test for all pairs of groups of patients. In this case, not only the ROC analysis of the areas under the ROC curve but also sensitivity and specificity for the best discriminators of liver functions and the breath test were compared. In most cases the 10th and 20th minute of PDR were found as the best discriminating time intervals. In our study the 20th minute of PDR as the best expression for the whole ^{13}C-MBT was selected (decision was based on sensitivity and specificity values of ROC analysis). Cut-off values of 20th minute of PDR for distinguishing between Controls and ChPA, ChPA and ChPB, and ChPB and ChPC were ≤ 27.39, ≤ 16.80 and ≤ 6.08, respectively (see Table 3 for details).

Comparison of the discriminating power of conventional liver tests, APRI score and AST/ALT ratio with the ^{13}C-MBT

In order to determine if there was a statistically significant difference in diagnostic power between the best routine liver test, AST/ALT ratio and the APRI score and the best time interval for the ^{13}C-methacetin breath test, Z-statistics defined by Hanley and McNeil were calculated. From the results shown in Table 3 it can be seen that in all cases the ^{13}C-methacetin breath test was more accurate and more sensitive than routine liver tests. However, specificities for routine liver tests were better in most cases. Statistically significant differences in AUCs between routine tests and the ^{13}C-MBT were found in only a few cases (statistically significant differences are marked in bold in Table 3). Both tests, the AST/ALT ratio and APRI score failed to differentiate between ChPB and ChPC groups of patients; in all other cases (Controls vs. ChPC, ChPA vs. ChPC and Controls vs. Cirrhosis) both, the AST/ALT ratio and APRI score were shown to have statistically less discriminating power relative to the best discriminating time interval for the ^{13}C-MBT.

DISCUSSION

The liver is the main site of alcohol metabolism. Alcohol is eliminated from the body through various metabolic mechanisms. The primary enzymes involved are aldehyde dehydrogenase (ALDH), alcohol dehydrogenase (ADH), cytochrome P450 (CYP2E1), and catalase[22]. Alcohol has a pronounced effect via ADH by changing the NADH/NAD ratio (reduced nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide ratio) in the mitochondria. When alcohol is metabolized to acetaldehyde, NAD is reduced to NADH and thus NAD is not available for the metabolism of ^{13}C-methacetin. Therefore, $^{13}CO_2$ production is decreased[23].

Variations in genes encoding ADH and ALDH produce alcohol- and acetaldehyde-metabolizing enzymes that vary in activity. This genetic variability influences a person's susceptibility to developing alcoholism and alcohol-related tissue damage, and alcohol dependence. Factors which cause variations in the rate of alcohol absorption, distribution, and elimination and which contribute significantly to clinical conditions observed after chronic alcohol consumption include environmental factors, gender, drinking pattern, fasting or fed states, and chronic alcohol consumption[24]. All these factors are the main sources of variability in the measurements of liver metabolic function among patients with chronic alcoholic liver disease and of the wide overlap in measured values particularly among healthy controls and patients in the early stage of the disease. Since the direct association between alcohol metabolism and liver's ability to produce $^{13}CO_2$ has been confirmed, we tried to determine, in our study, whether the ^{13}C-methacetin test can reliably reflect the complete extent of liver damage caused by alcohol consumption, from early stages to advanced liver cirrhosis[23,25].

Important findings of our study include: (i) patients with cirrhosis and healthy controls significantly differed in the ^{13}C-methcetin breath test throughout all time intervals; (ii) in addition the ^{13}C-MBT was able to differentiate patients with liver cirrhosis, relative to severity of hepatic impairment, corresponding to the Child-Pugh classification A vs. B vs. C and also between patients in the early stages of the liver disease (Child-Pugh A) and healthy controls. These observations are supported by data of Becker[14], Klatt[6] and Liu[26].

In clinical practice, liver metabolic functions are assessed using a complex evaluation of physical findings, biochemical tests and imaging procedures. There are many simple models based on routinely available biochemical test results (APRI score and AST/ALT ratio), which try to predict fibrosis and liver cirrhosis in patients with chronic liver diseases with various etiologies[27-29]. Obviously, such an assessment is not sensitive enough for evaluation of the complex processes that take place in

hepatocytes (biosynthesis, biotransformation, metabolism of xenobiotics[30] and are very unreliable. In our study we compared the APRI score, the AST/ALT ratio and serum bilirubin levels with the ^{13}C-MBT. (iii) The ^{13}C-MBT turned out to be superior to all, to the APRI score, to the AST/ALT ratio and to bilirubin in identifying patients with varying degrees of liver impairment.

For all pairs of groups we found (using ROC analysis) a time interval for the ^{13}C-MBT with sufficient sensitivity, specificity and overall accuracy. The ROC curve analysis suggests that the best prediction was provided by time intervals between the 10th - 20th minute of PDR. As for the clinical practice, just one single parameter is suitable, the 20th minute of PDR was selected and appropriate cut-off values between adjacent groups of patients, i.e. between Controls and ChPA, ChPA and ChPB and ChPB and ChPC, respectively, were calculated. This finding, which is in agreement with some studies[31,32,15] but not others[33], is of practical importance, since it suggests that further (up to 20 min) breath samples are not necessary. An exception found was in the discrimination between healthy controls and patients with nonsymptomatic cirrhosis (Child-Pugh A classification), which occurred at the 80th minute of PDR (the exception was based on the ROC analysis). Consequently, it seems that breath test could not be shortened, when patients in early stages of liver disease are expected in study samples. All our conclusions were drawn from a relatively small sample size of patients. For confirmation large, well-designed, prospective, longitudinal studies are needed.

Methacetin has numerous advantages compared with other substrates for liver function breath testing. Aminopyrine is the best investigated hepatic ^{13}C-labeled substrate so far, but it has a slow clearance rate and its use includes the risk of serious adverse events, such as agranulocytosis[34]. The ^{14}C-aminopyrine breath test has never found wide application because of the limits associated with a radioactive isotope. The newer methods such as the cocktail approach (testing the metabolic activity of several enzymes) (ref.[35]) and the analysis of several metabolites from a single test substance (metabolic fingerprint approach) (ref.[36]) have not found use in clinical hepatology because of inherent complexity and questionable reliability.

^{13}C-MBT is more time and labor consuming than the conventional biochemical tests. But, it is clear that such tests are superior to all routine liver biochemical tests as well as to the traditional Child-Pugh score in predicting survival or life-threatening complication. Moreover the ^{13}C-MBT can be used over the whole range of liver disease with widely varying severity, whereas the Child-Pugh score is applicable in hepatic cirrhosis only. Another important advantage of methacetin is its quick metabolism and safety profile. Additionally, innocuous methacetin is less expensive than a liver biopsy as well as other ^{13}C substrates which can be used for hepatic functional reserve assessment. Thus, for cirrhotic patients with an alcohol etiology, who are very often unable to undergo or who very often reject liver biopsy, the ^{13}C-MBT may prove to be a valuable tool for the diagnosis of advanced fibrosis and determination of the most appropriate treatment.

CONSLUSION

In conclusion, the ^{13}C-methacetin breath test can establish the diagnosis of liver cirrhosis with higher accuracy than any single biochemical marker and offers a reliable means for quantification of hepatic metabolic function over the complete range of liver functional impairment. It is non-invasive, easy to perform and completely safe. The methacetin breath test is a valuable addiction to clinical hepatologist´s array of liver assessment procedures.

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ABBREVIATIONS

ADH, Alcohol dehydrogenase; ALDH, Aldehyde dehydrogenase; ALT, Alanine aminotransferase; APRI, Aspartate aminotransferase to platelet ratio; AST, Aspartate aminotransferase; BILI, Serum bilirubin; ^{13}C-MBT, ^{13}C-methacetine breath test; CPDR, Cumulative percentage of dose rate; NAD, Nicotinamide dehydrogenase; NADH, reduced nicotinamide dehydrogenase; PDR, Percentage of dose rate; ROC, Receiver operating characteristic.

CONFLICT OF INTEREST STATEMENT

Author's conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

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