

Methionine Breath Test for Measuring Hepatic Mitochondrial Function

Name: ^{13}C -Methionine Breath Test

Background

Standard serologic and biochemical serum liver tests have been used to determine the presence of liver disease. However, these tests do not provide an accurate assessment of hepatic functional capacity nor do they detect changes in hepatic disease severity. To address this need, Metabolic Solutions, Inc. (MSI) has developed a non-invasive breath test that determines the degree and progression of liver impairment in patients with varied causes of liver disease. The test is based on the metabolism of $1\text{-}^{13}\text{C}$ methionine by hepatic mitochondria.

Overview

Liver disease affects 25 to 30 millions of Americans. There is a long latency period (5-30 years) until serious consequences occur like cirrhosis. Cirrhosis of the liver is the eighth leading cause of death by disease in the United States, killing 25,000 people each year. During the process leading to cirrhosis, liver cells are replaced by scar tissue, causing decreased hepatic function. The most common causes of cirrhosis are excessive alcohol use and chronic viral infection. However, recent evidence points to another major cause of cirrhosis, namely Non-Alcoholic Steato Hepatitis (NASH).

Hepatitis C virus (HCV) affects between 1 and 2 percent of Americans and chronic infection with HCV is probably the single most important cause of chronic liver disease in the Western world. Many patients are asymptomatic and are only diagnosed when they are found to have abnormal liver tests following a blood donation or routine evaluation for another problem. Yet, chronic hepatitis C can be insidious and slowly progressive and lead to cirrhosis and liver failure after years or decades of infection.

At present, there are no specific means of prevention of hepatitis C, and the only therapy of proven benefit is alpha interferon. Interferon treatment, however, is far from satisfactory. Therapy is expensive, often poorly tolerated, and results in a favorable long-term response in only a minority of patients.

NASH is liver damage in patients without significant alcohol consumption. NASH is now recognized as a problem of obesity that causes accumulation of fatty deposits in the liver that may eventually progress to cirrhosis. It is estimated that 25% of the population in the U.S. are obese and the number is still increasing. It is further estimated that up to 70% of these individuals may have a fatty liver and are at risk for NASH.

With all the advancements in modern medicine, there still is no approved test to determine functional capacity of the liver. Many people including physicians have called standard liver blood tests, "liver function tests". However, these tests can only indicate

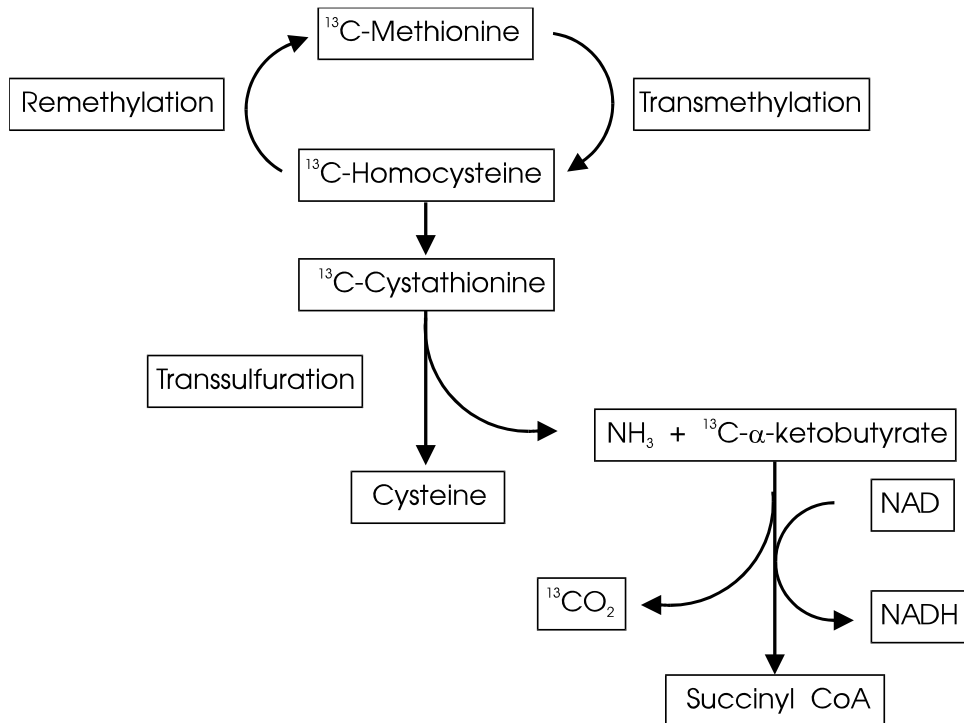
the presence of liver disease. These tests do not provide an accurate assessment of hepatic functional capacity nor do they detect changes in hepatic disease severity.¹ To address this need, Metabolic Solutions, Inc. has developed a non-invasive breath test that determines the degree and progression of liver impairment in patients with varied causes of liver disease. The test is based on the metabolism of 1-¹³C methionine by hepatic mitochondria and is called the methionine breath test (MBT).

The MBT represents an exciting new method for determining liver disease severity because it is a non-invasive, specific, and quantitative measure of hepatic mitochondrial function. Our studies demonstrate the effectiveness of the MBT in measuring hepatic mitochondrial function in individuals with liver disease.

Principle of the Methionine Breath Test:

Methionine is an essential amino acid that has important roles in various metabolic processes, including protein synthesis.^{2,3} The MBT uses ¹³C-methionine, which is a non-radioactive isotope and is metabolized exclusively by hepatic mitochondria. Subsequent to a dose, ¹³C-methionine metabolism results in an increased concentration of ¹³CO₂ in expired breath. The principle behind this technology is that the quantity of ¹³CO₂ measured in breath correlates with liver disease severity. The biochemistry of methionine metabolism is illustrated in figure 1.

Figure 1: ¹³C-Methionine Metabolism



Assessing Liver Function

Standard serum liver tests, radiological testing, and histological evaluation of liver biopsies do not measure liver function and do not predict prognostic outcomes. Increasing prothrombin time and decreasing serum albumin levels have been used as prognostic indicators of progressive liver disease.⁴ Significant changes in prothrombin time and albumin may occur in patients for reasons other than liver dysfunction and, at times, only after severe liver decompensation. Further, radiological testing and histological examinations of liver biopsies are poor indicators of decreasing hepatic function.

The Child-Pugh (CP) classification has been used to determine degree of liver disease severity. The CP classification reflects the sum of scores derived from clinical and laboratory parameters. Disadvantages of the CP classification include subjective measures (degree of ascites and encephalopathy) and dependence on serum tests (bilirubin, albumin, and prothrombin time) that may be influenced by extrahepatic factors. As a result, the CP classification is a poor measure of patient status and is insensitive to small, but significant, changes in the patient's condition.

Clinical Performance of the Methionine Breath Test

a) Reproducibility of the MBT

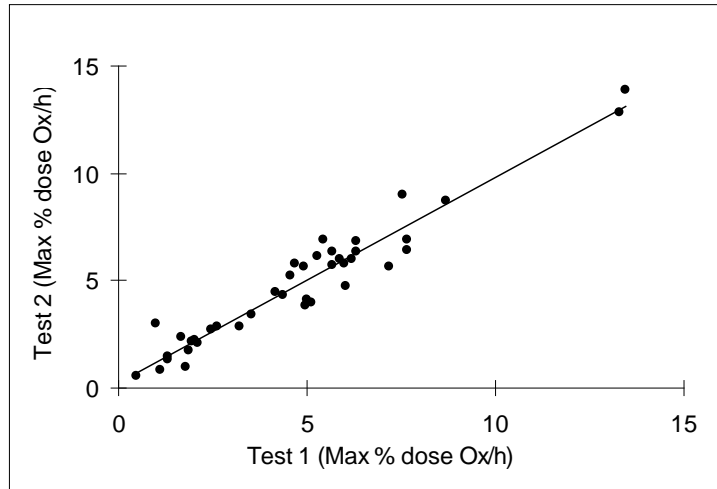
The objective of this study was to assess variability of the MBT by administering the test twice within 7 days to healthy volunteers and patients with previously diagnosed liver disease. The study included 20 healthy volunteers, 11 females and 9 males, with a mean age of 33 years (range 18-59). The 22 liver patients included (10 females and 12 males) and the mean age was 51 years (range 26-71). The liver patients included 11 patients with stable cirrhosis with no alcohol intake for > 1 year, 5 actively drinking alcoholic cirrhotic patients, and 6 patients with cirrhosis and alcoholic hepatitis. The MBT was performed while participants were at rest after an 8 hour fast. Reproducibility of the tests was evaluated using a paired Student's t-test. A linear correlation coefficient was also calculated for the two tests. The null hypothesis was that the difference between the two tests was equal to zero. The null hypothesis was tested at $p < 0.05$.

Reproducibility of the replicate tests was excellent as the paired t-test accepted the hypothesis that no difference was detectable between the tests ($p=0.735$). The raw data is shown in table 1. There was a significant correlation ($r = 0.966$, $p < 0.001$) between MBT test 1 and test 2. The correlation plot is given in figure 2. These results show the reproducibility of the MBT over a broad clinical range of liver function.

Table 1: Replicate MBT tests in healthy controls and patients with liver disease. Results expressed as maximum percent dose oxidized per hour.

Participant	Test 1	Test 2
1	6.18	6.01
2	13.28	12.81
3	5.29	6.14
4	7.64	6.44
5	4.56	5.21
6	4.16	4.47
7	6.02	4.72
8	13.44	13.87
9	5.45	6.88
10	7.55	9.00
11	6.01	5.82
12	4.97	3.87
13	6.31	6.33
14	7.66	6.93
15	5.69	6.34
16	8.71	8.72
17	5.86	5.98
18	5.11	3.98
19	4.91	5.62
20	4.67	5.78
P1	5.01	4.09
P2	6.30	6.81
P3	2.47	2.69
P4	7.18	5.68
P5	5.66	5.71
P6	3.20	2.84
P7	3.55	3.39
P8	1.67	2.39
P9	4.36	4.31
P10	2.63	2.83
P11	2.10	2.11
P12	0.99	2.98
P13	2.02	2.24
P14	1.31	1.33
P15	1.87	1.77
P16	1.94	2.14
P17	0.46	0.59
P18	1.80	0.99
P19	1.11	0.82
P20	1.31	1.44

Figure 2: MBT Test 1 vs Test 2 data for healthy controls and liver patients.

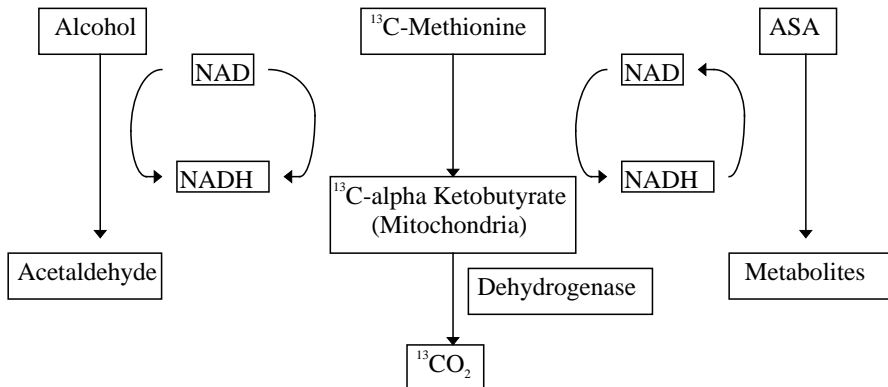


We found that the reproducibility of the MBT was excellent in 20 volunteers as well as in the 20 patients with liver disease. Short-term reproducibility proved satisfactory, as differences between test 1 and test 2 were not significantly different from zero at 95% confidence with a paired t-test. Correlation analysis yielded ($r = 0.966$, $p < 0.001$) across the entire clinical range.

b) Modulation of Mitochondrial Function

We explored how the MBT responded to agents that effect mitochondrial function. Alcohol and aspirin (ASA) have a pronounced effect on changing the NADH/NAD ratio in the mitochondria. How the change of the NADH/NAD ratio affects methionine metabolism is illustrated in figure 3.

Figure 3: Effect of alcohol and Aspirin on methionine metabolism



When alcohol or aspirin change the NAD/NADH ratio this either decreases or increases methionine oxidation, respectively. When alcohol is metabolized to acetaldehyde, NAD is reduced to NADH and thus NAD is not available for the metabolism of ^{13}C -

methionine. Therefore, $^{13}\text{CO}_2$ production is decreased. When ASA is metabolized, NADH is oxidized to NAD making more NAD available for methionine metabolism. Therefore, $^{13}\text{CO}_2$ production is increased.

Initially 20 healthy volunteers were evaluated with the MBT. Then, the next day, participants were randomized to ingest either alcohol (n=9) or aspirin (n=11) 15 minutes prior to the MBT. Subjects ingested 60 cc (2 oz) vodka (Smirnoff, 86 proof) dissolved in 200 ml orange juice. The dose of aspirin ingested was 30 mg/kg body weight (5-8 tablets) and was swallowed with 100 ml water. A paired Student t-test compared the mean of MBT tests 1 and 2 to the MBT performed during the ingestion of alcohol or aspirin.

Data for the two treatment groups is shown in table 2. The mean maximum % oxidation per hour for all subjects was 6.71 ± 2.55 (mean \pm 1 SD). In subjects who ingested alcohol, the MBT decreased to 3.01 ± 0.81 (mean change from baseline $-59 \pm 17\%$, $p < 0.003$) and in those who ingested aspirin, the MBT increased to 8.43 ± 1.16 (mean change from baseline $36 \pm 36\%$, $p < 0.001$).

Table 2: Effect of alcohol and ASA ingestion on the MBT in healthy controls. Results are expressed as maximum percent dose per hour.

MBT Group 1			MBT Group 2		
Participant	Baseline	Alcohol	Participant	Baseline	ASA
1	6.10	3.36	10	8.28	9.47
2	13.05	4.54	11	5.92	10.08
3	5.72	2.76	12	4.42	8.88
4	7.04	2.22	13	6.32	8.73
5	4.89	2.97	14	7.30	7.77
6	4.32	3.39	15	6.02	7.34
7	5.37	1.86	16	8.72	9.24
8	13.66	3.52	17	5.92	6.47
9	6.17	2.45	18	4.55	8.61
			19	5.27	6.88
			20	5.23	9.27
Mean	7.37	3.01		6.17	8.43
SD	3.48	0.81		1.41	1.16

The results indicate that the MBT significantly decreases with ingestion of alcohol and increases with aspirin ingestion. Changes in the MBT, therefore, reflect changes in hepatic mitochondrial function. This study suggests that the MBT is a sensitive and specific measure of changes in hepatic mitochondrial function induced by alcohol and aspirin and can be used as a quantitative measure of mitochondrial function.

c) Ability of the MBT to detect Liver Disease

The goal for this experiment was to determine if the MBT is capable of distinguishing healthy controls from patients with liver disease. We studied 27 healthy controls and 46 patients with well-characterized cirrhosis. All patients were diagnosed with cirrhosis based on clinical symptoms, standard serum liver tests, radiological testing, and/or liver histology. All patients were clinically stable. The current practice guideline is to determine liver disease severity using the Child-Pugh (CP) classification that uses standard liver blood test results and presence of other symptoms. The CP score was determined on all liver patients at the time of the first test and were calculated as shown in table 3.

Table 3: Child-Pugh scoring and classification system.

Points	Prothrombin Time (seconds over control)	Albumin (g/dl)	Total Bilirubin (mg/dl)	Ascites	Encephalopathy (grade)
1	<4	>3.5	<2	None	0
2	4-6	3.5-2.8	2-3	Mild	1-2
3	>6	<2.8	>3	Moderate/Severe	3-4

CP Classification	CP Score (total Points)
A	5-6
B	7-9
C	10-15

Differences between healthy controls, CP A, CP B, and, CP C were compared using a One Way Analysis of Variance with Bonferroni Group Mean Comparisons. Comparisons were made between groups as follows; healthy controls and CP A, CP A and CP B, and CP B and CP C.

Analysis of the data showed that the MBT distinguished healthy controls and patients with different degrees of liver disease severity. Healthy controls (9.16 ± 2.62 , mean \pm 1 SD) had MBT results that were significantly different ($p < 0.001$) from liver patients characterized as CP A (4.10 ± 4.53). Liver patients classified as CP A (4.10 ± 4.53) were significantly different ($p = 0.003$) from CP B (2.57 ± 1.37) patients. CP B patients had MBT values that were less dramatic but still significantly different ($p = 0.02$) from CP C (1.33 ± 0.76) patients.

Using the threshold of 6% (maximum % dose oxidized /h) patients were classified as either having normal or abnormal mitochondrial function. Abnormal function was classified as an MBT $< 6\%$ and normal function if $\geq 6\%$. Utilizing the threshold of 6%, sensitivity was 96%, specificity 100%, and accuracy 97% (see figure 4 and table 4).

Figure 4: MBT in controls and patients showing the threshold level of 6% differentiates controls from liver patients classified as CP A, CP B, or CP C.

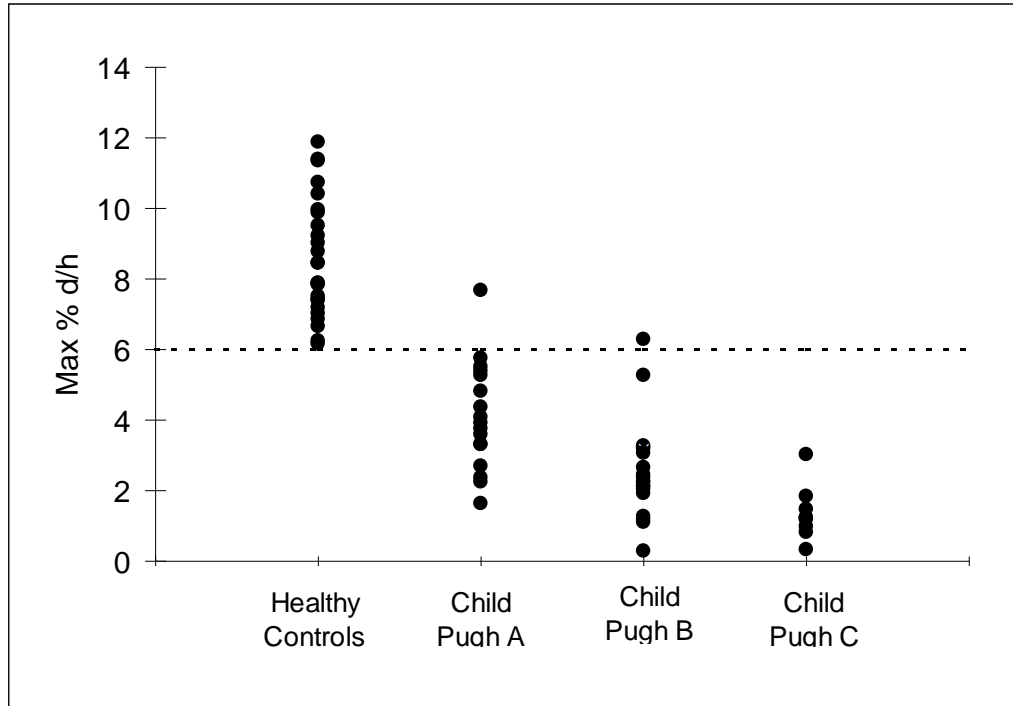


Table 4: Sensitivity and Specificity of the MBT for all participants.

MBT (%)	Child Pugh A, B, C	Controls
< 6.0	44 Total Positive	0 False Positive
≥ 6.0	2 False Negative	27 True Negative

The sensitivity, specificity, and accuracy of the test were calculated as follows:

$$\begin{aligned} \text{Sensitivity} &= \text{No. of True Positives} / \text{Total with Disorder} \\ &= 44 / 46 \times 100 = 96\% \end{aligned}$$

$$\begin{aligned} \text{Specificity} &= \text{No of True Negatives} / \text{Total free of Disorder} \\ &= 27 / 27 \times 100 = 100\% \end{aligned}$$

$$\begin{aligned} \text{Accuracy} &= \text{True Positives} + \text{True Negatives} / \text{Total Investigated} \\ &= [(44+27) / 73] \times 100 = 97\% \end{aligned}$$

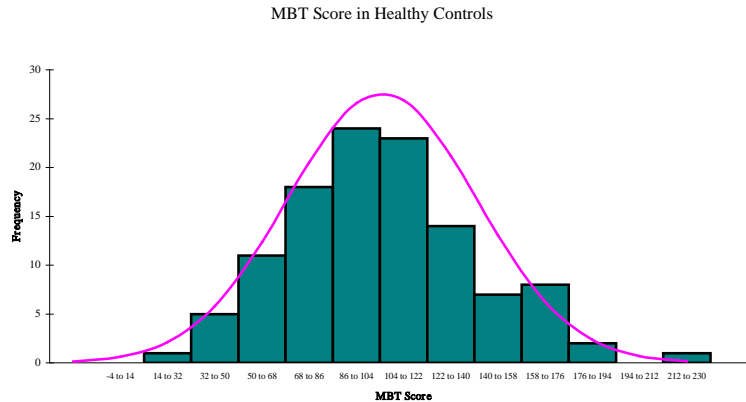
The MBT was able to distinguish healthy controls, CP A, CP B, and CP C patients with high specificity and sensitivity. Our data suggests that mitochondrial function is compromised as liver disease progresses either through the CP scoring system or from stable cirrhosis to alcoholic hepatitis. In addition, serial testing of patients over time may be useful as a prognostic indicator of liver disease progression and provide information for optimal timing of therapeutic measures including liver transplantation. Serial testing may indicate a response to treatment and therefore allow the clinician to employ a cost effective treatment strategy by either continuing useful therapy or discontinuing ineffective therapy.

It was not surprising that some overlap of the MBT results occurred for each of the Child's classifications. This is predicted by existing literature that reported CP A patients have a wide range of hepatic functional impairment ranging from nearly normal to severely abnormal. The Child's score is only for classification purposes and is not a true measure of liver function. The Child's classification should not be considered as a reference test of liver function. In fact, the MBT shows some patients with CP A and CP B have normal function. The clinical occurrence of normal liver function in a CP A or CP B occurs frequently. This is the reason that the Child's scoring does not offer the clinician insight into quantitative liver function reserves.

d) Determining Normal Range of the MBT

One hundred and fifty (150) individuals without liver disease were administered the MBT to understand the inter-subject variation and normal range of the test. Subjects were determined to be free of liver disease if they had normal liver chemistries, no blood alcohol levels prior to testing, no Hepatitis C antigens, and no substance abuse in the urine.

The mean and median rate of methionine oxidation was 4.00% per hour in this larger group of subjects. We felt it was necessary to revise the scoring of the test because the differences in the numbers were very small. We have converted the methionine oxidation rate to a "MBT Score" by multiplying all results by 25. This made the results easy to interpret and easier to inspect true differences between healthy subjects and those with liver disease. Therefore, the mean MBT Score is now 100. A histogram of the results revealed a normal distribution as shown below.



e) Establishment for a cutoff of Cirrhosis by the MBT

A retrospective analysis of our database was used to define a MBT cutoff value that would predict the presence of cirrhosis. In the MBT analysis, 165 subjects without any known liver disease and 22 biopsy-proven cirrhotics were used in the analysis. Using ROC analysis, a MBT cutoff score of less than 38 was indicative of cirrhosis. The sensitivity of the MBT was 91% (20/22 correct) and the specificity was 98% (161/165). These results showed an overall accuracy of the MBT to predict cirrhosis at 97%. This level of accuracy is compelling to evaluate further whether the MBT can replace or reduce the use of liver biopsies.

A reduction in liver biopsies will not only reduce healthcare costs but also improve quality of life of patients. Liver biopsies are associated with pain in 30% of patients, severe complications in 0.3%, and death in 0.03%. It has also been reported that the duration of the pain after biopsy extends beyond the day of the biopsy in 40% of patients and extends for over 1 week in a small number.⁷ In fact, when questioned after the biopsy, 15% of the patients said that they would not have agreed to the procedure if they knew ahead how they would feel during and after the procedure.

f) Reduction of the Number of Breath Samples

We recently developed a way to reduce the number of breath samples taken with the MBT, which is currently every 10 minutes for one hour. Using a database of over 400 subjects, we have found an excellent correlation between the 40 minute breath collection and the area-under-the-curve for the entire hour. The correlation was found to be $r^2 = 0.986$, $p < 0.0001$. There was no statistical difference in the final MBT Score calculated with the one point (40 minute time point) method or the six point (every 10 minutes for 1 hour) method. Since the database is so large, we are convinced that the MBT can be performed with just one breath collection after administering the methionine dose.

Summary of experiments:

- 1) Reproducibility of the MBT was excellent in all participants with a correlation of

($r = 0.97$).

- 2) The statistically significant changes in the MBT after alcohol and aspirin ingestion, agents that are known to inhibit or induce mitochondrial metabolism respectively, indicate that the MBT monitors mitochondrial function.
- 3) MBT results were significantly different for healthy controls and patients classified as Child-Pugh A, B, and C. The MBT was also able to identify patients with normal and abnormal function with a high degree of sensitivity, specificity, and accuracy.
- 4) An average MBT Score is 100 and we have established a cutoff for cirrhosis of 38.
- 5) The MBT had an accuracy of 97% of predicting cirrhosis suggesting it was useful to reduce the number of liver biopsies performed.
- 6) We have established that the MBT can be performed with just two breath collections, a pre-breath sample and a 40 minute post-methionine dose sample.

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