H nuclear magnetic resonance spectroscopy-based metabonomic study in patients with cirrhosis and hepatic encephalopathy

Citation for published version:  

Digital Object Identifier (DOI):  
10.4254/wjh.v7.i12.1701

Link:  
Link to publication record in Edinburgh Research Explorer

Document Version:  
Publisher's PDF, also known as Version of record

Published In:  
World Journal of Hepatology

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Download date: 21. Sep. 2019
Randomized Clinical Trial

1H nuclear magnetic resonance spectroscopy-based metabonomic study in patients with cirrhosis and hepatic encephalopathy

Konstantinos John Dabos, John Andrew Parkinson, Ian Howard Sadler, John Nicholas Plevris, Peter Clive Hayes

Received: March 12, 2015
Peer-review started: March 12, 2015
First decision: April 10, 2015
Revised: May 9, 2015
Accepted: June 4, 2015
Article in press: June 8, 2015
Published online: June 28, 2015

Abstract

AIM: To identify plasma metabolites used as biomarkers in order to distinguish cirrhotics from controls and encephalopathics.

METHODS: A clinical study involving stable cirrhotic patients with and without overt hepatic encephalopathy was designed. A control group of healthy volunteers was used. Plasma from those patients was analysed using 1H - nuclear magnetic resonance spectroscopy. We used the Carr Purcell Meiboom Gill sequence to process the sample spectra at ambient probe temperature. We used a gated secondary irradiation field for water signal suppression. Samples were calibrated and referenced using the sodium trimethyl silyl propionate peak at 0.00 ppm. For each sample 128 transients (FID’s) were acquired into 32 K complex data points over a spectral width of 6 KHz. 30 degree pulses were applied with an acquisition time of 4.0 s in order to achieve better resolution, followed by a recovery delay of 12 s, to allow for complete relaxation and recovery of the magnetisation. A metabolic profile was created for stable cirrhotic patients without signs of overt hepatic encephalopathy and encephalopathic patients as well as healthy controls. Stepwise discriminant analysis was then used and discriminant factors were created to differentiate between the three groups.
RESULTS: Eighteen stabled cirrhotic patients, eighteen patients with overt hepatic encephalopathy and seventeen healthy volunteers were recruited. Patients with cirrhosis had significantly impaired ketone body metabolism, urea synthesis and gluconeogenesis. This was demonstrated by higher concentrations of acetocetate (0.23 ± 0.02 vs 0.05 ± 0.00, P < 0.01), and b-hydroxybutyrate (0.58 ± 0.14 vs 0.08 ± 0.00, P < 0.01), lower concentrations of glutamine (0.44 ± 0.08 vs 0.63 ± 0.03, P < 0.05), histidine (0.16 ± 0.01 vs 0.36 ± 0.04, P < 0.01) and arginine (0.08 ± 0.01 vs 0.14 ± 0.02, P < 0.03) and higher concentrations of glutamate (1.36 ± 0.25 vs 0.58 ± 0.04, P < 0.01), lactate (1.53 ± 0.11 vs 0.42 ± 0.05, P < 0.01), pyruvate (0.11 ± 0.03 vs 0.03 ± 0.00, P < 0.01), threonine (0.39 ± 0.02 vs 0.08 ± 0.01, P < 0.01) and aspartate (0.37 ± 0.03 vs 0.03 ± 0.01). A five metabolite signature by stepwise discriminant analysis could separate between controls and cirrhotic patients with an accuracy of 98%. In patients with encephalopathy we observed further derangement of ketone body metabolism, impaired production of glycerol and myoinositol, reversal of Fischer’s ratio and impaired glutamine production as demonstrated by lower b-hydroxybutyrate (0.58 ± 0.14 vs 0.16 ± 0.02, P < 0.0002), higher acetocetate (0.23 ± 0.02 vs 0.41 ± 0.16, P < 0.05), leucine (0.33 ± 0.02 vs 0.49 ± 0.05, P < 0.005) and isoleucine (0.12 ± 0.02 vs 0.27 ± 0.02, P < 0.0004) and lower glutamine (0.44 ± 0.08 vs 0.36 ± 0.04, P < 0.013), glycerol (0.53 ± 0.03 vs 0.19 ± 0.02, P < 0.000) and myoinositol (0.36 ± 0.04 vs 0.18 ± 0.02, P < 0.010) concentrations. A four metabolite signature by stepwise discriminant analysis could separate between encephalopathic and cirrhotic patients with an accuracy of 87%.

CONCLUSION: Patients with cirrhosis and patients with hepatic encephalopathy exhibit distinct metabolic abnormalities and the use of metabolomics can select biomarkers for these diseases.

Key words: Ketone bodies; Branch chain amino acids; Glutamine; Glycolysis

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Core tip: Few studies have approached the metabolic abnormalities of liver cirrhosis and its complication hepatic encephalopathy. This study provides evidence that in stable cirrhosis key metabolic pathways are impaired and confirms the fact that there is impaired gluconeogenesis, impaired ketogenesis and ketone bodies break down as well as impaired urea cycle. In encephalopathy there is a reversal in the pattern of branch chain amino acids concentrations towards normal. By using stepwise discriminating analysis we were able to separate with remarkable accuracy metabolic phenotypes of cirrhotic patients from controls and also those who suffered from encephalopathy from those cirrhotics who did not.


INTRODUCTION

Insults on the liver parenchyma could result in fat accumulation, inflammation and fibrosis. Most chronic liver injuries could result in cirrhosis, which is a combination of hepatic fibrosis and nodular regenerative hyperplasia. Hepatitis B infection is the most common cause in Asia and Africa whereas alcohol is the most common cause in the developed world. Currently worldwide, the proportion of cirrhosis due to chronic Hepatitis C infection is on the increase.[1,2]

One of the major complications of cirrhosis is hepatic encephalopathy. This is a complex neuropsychiatric syndrome which has a potential for full reversibility. It is characterised by global depression of the central nervous system (CNS) and has different degrees of severity. The syndrome is usually episodic and relapsing but some patients exhibit a chronic protracted course.[3]

Cirrhosis is associated with alterations in proteins and amino acids metabolism, including diminished urea formation and hyperammonaemia.[4,5]. Most studies that have looked at amino acid metabolism in cirrhosis would agree that there seems to be a recognisable pattern in the plasma amino acid profile with an elevation of aromatic amino acids (AAA) and methionine and reduced levels of branch chain amino acids (BCAA).[6,7]. Although this pattern has been used as the basis for the false neurotransmitter theory in the pathogenesis of hepatic encephalopathy,[8] not many studies have proved that the pattern observed in cirrhosis is valid for encephalopathy too.[6,9,10]. Furthermore it is now well accepted that encephalopathy due to acute hepatic failure is a different entity and the mechanisms contributing to the CNS dysfunction in the two diseases might be different.

Apart from disturbances in the BCAA to AAA ratio, other biochemical abnormalities are present in cirrhotics. Gluconeogenesis is impaired and hyperammonaemia and diminished urea production make it necessary for the body to find other pathways for nitrogen elimination.[5,11,12].

In the last few years, the emerging field of metabolomics, which examines global metabolic profiles using various data collection techniques, offered the possibility to identify biomarkers in evolving diseases.[13]. Lately, this technique has been used in delineating disease phenotypes in humans suffering from chronic liver disease.[14,15] and animal models of liver failure.[16,17]. Metabolomics makes use of multivariate statistical approaches to analyse complex data sets, such as those obtained by "H NMR spectroscopy.[16]. It is particularly useful if there are few samples to analyze and many variables to consider.

The aim of our study was to apply "H NMR spec-
troscopy in controls, cirrhotics and cirrhotics with hepatic encephalopathy and analyse the data to identify metabolic patterns that could distinguish between the three groups.

**MATERIALS AND METHODS**

**Patients**
The Local Research Ethics committee had approved the study protocol (373/1997). The study protocol conformed to the guidelines of the 1975 Declaration of Helsinki. Patients were recruited at the Centre of Liver and Digestive Disorders and at the Day Case Unit at the Royal Infirmary of Edinburgh. All patients signed an informed consent. For those unable to consent due to encephalopathy, consent from the next of kin was sought. Encephalopathy was defined using the West Haven Criteria[9]. The diagnosis of cirrhosis was based on a combination of clinical, histopathological and imaging criteria. Patients with grade III or IV hepatic encephalopathy were excluded from the study as they usually had an acute precipitating episode and were not stable cirrhotics. Patients with coagulation abnormalities (International Normalized Ratio < 2.0, platelets < 80000) were excluded from the study as the ethics committee did not approve internal jugular vein puncture in patients with impaired coagulation. Patients with an upper gastrointestinal bleeding episode in the previous two weeks were also excluded. Patients with hepatocellular carcinoma, pregnant and lactating women were also excluded from the study.

A total of 42 patients were recruited. Six of them eventually withdrew consent and were not included in the study. The study recruited for just over 16 mo.

We studied patients with stable cirrhosis (group A, 18 patients), patients with stable cirrhosis during an episode of hepatic encephalopathy (group B, 18 patients) and sex and age matched normal controls (group C, 17 subjects).

All patients were fasted overnight before blood collection. Blood was collected from the internal jugular vein, from all three groups two to three hours following a main meal as differences in the concentration of amino acids between sexes are less pronounced postprandially, in lithium heparin tubes. It was immediately centrifuged at 2000 g for 15 min at 4 °C. The supernatant was then aliquoted in 2.5 mL vials. The vials were then stored at -40 °C until NMR analysis.

**Sample preparation for NMR spectroscopy**
Samples were prepared by adding a D:O solution (150 μL) to plasma (600 μL) providing an internal field frequency lock for the spectrometer, a Varian 600 MHz at 14.1T. Five millimeter probes were used for the analysis. Chemical shifts were referenced externally to the singlet methyl resonance of sodium trimethyl silyl propionate (TSP) (75 μL) at zero ppm. Plasma samples were left in room temperature for 1 h before samples for the NMR analysis were prepared.

**Proton NMR spectroscopy**
The CMPG sequence was applied, to acquire our data, as this sequence enabled us to observe a flat baseline in our spectra from plasma samples, by minimising the signals acquired from macromolecules present in the plasma such as proteins and lipoproteins. All spectra were acquired at ambient probe temperature (298 ± 0.2 K). For each sample 128 transients (FID’s) were acquired into 32 K complex data points over a spectral width of 6 KHz. 30° pulses were applied with an acquisition time of 4.0 s in order to achieve better resolution, followed by a recovery delay of 12 s, to allow for complete relaxation and recovery of the magnetisation. Water signal suppression was achieved by applying a gated secondary irradiation field at the water resonance frequency.

**Spectral processing**
FID’s were multiplied by an exponential function before applying Fourier transform. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated using the TSP peak at 0.00 ppm. A preliminary assignment of the amino acid metabolites was performed and only the areas between 0.70 and 3.80 ppm and between 6.80 and 7.70 ppm were subjected to stepwise discriminant analysis (SDA).

To assess which peaks were significantly different between the three groups a one-way analysis of variants was used. Normality of data distribution was assessed using the Wilk’s Lambda distribution.

Spectral assignments were made by reference to literature values of chemical shifts in various media and biological fluids (18) and coupling constants. Spectra were processed using the Mestre-C software (Mestrelab, Santiago de Compostela, Spain).

**Variables**
We measured a large array of aminoacids and products of cellular metabolism to ensure representation of the main metabolic pathways performed by the hepatocyte in our results. The following substances were measured. Lactate, pyruvate, acetoacetate, β-hydroxybutyrate, leucine, isoleucine, valine, alanine, threonine, glycine, aspartate, glutamine, glutamate, histidine, arginine, methylamine, dimethylamine, trimethylamineoxide (TMAO), glycerol, and myoinositol. Results are expressed as mmols/L unless otherwise state.

**Statistical analysis**
To compare between the three groups we used the three way ANOVA test. Where the ANOVA test was statistically significant the Tuckey test was performed to compare between groups. Values are expressed as mean (range and standard error). A P value of < 0.05 was taken as statistically significant (two-tail test of significance).

For the multivariate analysis we opted for the SDA. Data with statistical significance on ANOVA were entered into the SDA. We used SDA to extract and classify variables from different spectra. Analysis was performed
Patients with hepatic encephalopathy had more severe liver disease. CP: Child-Pugh; M: Male; F: Female.

Table 1 Results for glycolysis end products and gluconeo­genic precursors are shown

<table>
<thead>
<tr>
<th>Chemical shift</th>
<th>Lactate</th>
<th>Pyruvate</th>
<th>Alanine</th>
<th>Threonine</th>
<th>Glycine</th>
<th>Aspartate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1.33</td>
<td>2.38</td>
<td>1.48</td>
<td>1.34</td>
<td>3.57</td>
<td>2.82</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>1.53±0.11</td>
<td>0.11±0.02</td>
<td>0.77±0.04</td>
<td>0.39±0.02</td>
<td>0.31±0.03</td>
<td>0.37±0.03</td>
</tr>
<tr>
<td>Encephalopathy</td>
<td>1.41±0.13</td>
<td>0.17±0.02</td>
<td>0.73±0.06</td>
<td>0.25±0.01</td>
<td>0.18±0.01</td>
<td>0.27±0.02</td>
</tr>
<tr>
<td>Controls</td>
<td>0.42±0.05</td>
<td>0.03±0.00</td>
<td>0.61±0.05</td>
<td>0.08±0.01</td>
<td>0.09±0.01</td>
<td>0.03±0.01</td>
</tr>
</tbody>
</table>

Lactate, pyruvate, alanine, threonine, glycine and aspartate concentrations were all significantly higher in patients than controls (P < 0.01 in all cases).

Table 2 Results for ketone bodies, branch chain and aromatic amino acids are shown

<table>
<thead>
<tr>
<th>Chemical shift</th>
<th>Acetoacetate</th>
<th>B-hydroxybutyr</th>
<th>Leucine</th>
<th>Isoleucine</th>
<th>Valine</th>
<th>Phenylalanine</th>
<th>Tyrosine</th>
<th>Methionine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>2.29±0.02</td>
<td>2.31±0.14</td>
<td>0.96±0.02</td>
<td>1.01±0.02</td>
<td>1.04±0.01</td>
<td>7.38±0.01</td>
<td>6.91±0.02</td>
<td>2.14±0.02</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>0.23±0.02</td>
<td>0.58±0.14</td>
<td>0.33±0.02</td>
<td>0.12±0.02</td>
<td>0.14±0.01</td>
<td>0.08±0.01</td>
<td>0.23±0.06</td>
<td>0.07±0.00</td>
</tr>
<tr>
<td>Encephalopathy</td>
<td>0.41±0.05</td>
<td>0.16±0.02</td>
<td>0.49±0.05</td>
<td>0.27±0.02</td>
<td>0.16±0.02</td>
<td>0.06±0.02</td>
<td>0.25±0.06</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td>Controls</td>
<td>0.05±0.00</td>
<td>0.08±0.00</td>
<td>0.35±0.02</td>
<td>0.13±0.02</td>
<td>0.36±0.03</td>
<td>0.02±0.01</td>
<td>0.07±0.00</td>
<td>0.03±0.01</td>
</tr>
</tbody>
</table>

Acetoacetate and B-hydroxybutyrate concentrations were significantly higher in patients than controls (P < 0.01 in all cases). Aromatic amino acids concentrations were significantly higher in patients than controls (P < 0.01 in all cases). Valine concentrations were significantly lower in patients than controls (P < 0.01). Leucine was significantly higher if we compared encephalopathics with controls (P < 0.01), but there was no difference if we compared cirrhotics and controls. Isoleucine was significantly lower if we compared encephalopathics with controls (P < 0.01) but there was no difference between cirrhotics and controls.

Table 3 Results for urea cycle intermediates are shown

<table>
<thead>
<tr>
<th>Chemical shift</th>
<th>Glutamine</th>
<th>Glutamate</th>
<th>Histidine</th>
<th>Arginine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>2.46</td>
<td>2.36</td>
<td>7.83</td>
<td>1.93</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>0.44±0.08</td>
<td>1.56±0.25</td>
<td>0.16±0.01</td>
<td>0.08±0.01</td>
</tr>
<tr>
<td>Encephalopathy</td>
<td>0.36±0.04</td>
<td>0.84±0.16</td>
<td>0.18±0.02</td>
<td>0.13±0.02</td>
</tr>
<tr>
<td>Controls</td>
<td>0.63±0.03</td>
<td>0.58±0.04</td>
<td>0.36±0.04</td>
<td>0.14±0.02</td>
</tr>
</tbody>
</table>

Glutamine, histidine and arginine concentrations were significantly lower in patients than controls (P < 0.01 in all cases). Glutamate concentrations were significantly higher in cirrhotics (P < 0.01) compared to controls. It was also significantly increased if we compared encephalopathics with controls (P < 0.05).

Table 4 shows the results obtained for urea cycle end products. Glutamine, histidine and arginine concentrations were significantly lower in patients than controls (P < 0.01 in all cases). Glutamate concentrations were significantly higher in cirrhotics (P < 0.01) compared to controls. They are also significantly increased if we compared encephalopathics with controls (P < 0.05).

Table 5 shows the results for methylamine, dimethylamine, TMAO, glycerol and myoinositol. Methylamine, dimethylamine and TMAO concentrations were present in similar amounts in cirrhotic and encephalopathic patients but were absent in controls. Glycerol concentrations were significantly higher in patients than controls (P < 0.01 in both cases). Myoinositol concentrations were significantly higher in cirrhotics (P < 0.015) but there were no differences between encephalopathic patients and controls.

Using SDA we were able to identify five metabolites, tyrosine, phenylalanine, methionine, pyruvate and glycine that yielded the strongest segregation between groups A and C. A discriminant function (sum of concentrations of all five metabolites (tyrosine + phenylalanine + methionine + pyruvate + glycine) in mmols/L < 0.50 for controls) was created. By performing ROC analysis it had

**RESULTS**

Patient characteristics in groups A and C are shown in Table 1. Patients were well matched for age and sex. Patients with hepatic encephalopathy had in general more severe liver failure.

Table 2 shows the results for ketone bodies, BCAA and AAA. Acetoacetate and B-hydroxybutyrate, tyrosine, phenylalanine and methionine concentrations were all significantly higher in patients than controls (P < 0.01 in all cases). Valine was significantly lower in patients than controls (P < 0.01 in both cases). Leucine was significantly higher in encephalopathics than controls (P < 0.01), but there was no difference between cirrhotics and controls. Isoleucine was significantly lower in cirrhotics (P < 0.01) but there was no difference between cirrhotics and controls.

Table 3 shows the results obtained for glycolysis. Lactate and pyruvate concentrations were significantly higher in patients than controls (P < 0.01 in all cases). Alanine, threonine, glycine and aspartate concentrations were significantly higher in patients than controls (P < 0.01 in all cases).

In stepwise manner entering variables with the highest statistical significance first. A discriminant function was thus established and receiver operator curves (ROC) analysis was performed. Analyses were performed using SAS 8.0 software (SAS Institute, Cary, NC, United States).
Metabolites were uniformly increased. It appears then that gluconeogenesis is generally impaired in cirrhosis and encephalopathy. This would be in accordance with previous studies in humans and animal models. Pyruvate and glycine were part of the discriminant function between cirrhosis and healthy controls.

Our study showed that the concentrations of ketone bodies were significantly increased in both groups of patients compared to controls. In encephalopathies, acetocacetate was even more increased than in cirrhosis but β-hydroxybutyrate concentrations were decreased. The fact that all ketogenic amino acids are increased in cirrhosis as well, would favour a hypothesis of impaired ketone bodies utilisation in the periphery (muscle, brain). The fact that β-hydroxybutyrate and acetate are significantly decreased in encephalopathic cirrhotics is indicative of an impaired ketogenesis. We observed, however, that acetocacetate is increased in encephalopathies. Acetoacetate is the main product of ketogenesis and then by using nicotinamide adenine dinucleotide hydrogen (NADH) as co-substrate is further metabolised to acetate in the cellular mitochondria. β-hydroxybutyrate was part of the main discriminant function between cirrhotic and encephalopathic patients. We can hypothesize that, possibly, the precarius state of energy production in encephalopathy makes the availability of NADH for this further reaction minimal and it is shifted towards energy production from the Kreb's cycle, which is vital to the hepatocytes, instead of finalising a product which is destined for export to other organs like muscle and brain. This is further consolidated by the fact that encephalopathics were shown to have a significantly lower glycerol level. This is an indication that fewer triglycerides are broken down and fewer lipids are made available for oxidation which is the main pathway that would lead to ketone body production. Glycerol was part of the main discriminant function between cirrhotic and encephalopathic patients. This lends support to the recent hypothesis that the phenylacetate could be used as a treatment in hepatic encephalopathy and to subsequent studies in animal models that were in accordance with that.

Typical changes in plasma amino acid patterns have been found in different studies in patients and experimental animals in chronic liver failure. Those changes are increased concentrations of the AAA and methionine and decreased concentrations of the BCAA. The AAA and methionine are primarily metabolised by the liver and their raised concentrations in both cirrhotics and encephalopathic cirrhotics are probably due to impaired liver metabolism and portosystemic shunting of blood. Our findings related to AAA confirm findings by numerous previous studies which showed an increase in AAA concentrations.

The story is more complex for the BCAA and is further complicated by the findings of this study that in encephalopathies there was an increase in the concentrations of leucine and isoleucine. The normal liver does not play a major role in the breakdown of the BCAA which are mostly catabolised in the skeletal muscle

<table>
<thead>
<tr>
<th>Chemical shift</th>
<th>Cirrhotics</th>
<th>Encephalopathics</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylamine</td>
<td>2.54 ± 0.03</td>
<td>1.9 ± 0.03</td>
<td>0</td>
</tr>
<tr>
<td>Dimethylamine</td>
<td>2.72 ± 0.03</td>
<td>3.1 ± 0.04</td>
<td>0</td>
</tr>
<tr>
<td>Tmao</td>
<td>3.27 ± 0.07</td>
<td>5.1 ± 0.08</td>
<td>0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>3.79 ± 0.09</td>
<td>2.0 ± 0.08</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Myoinositol</td>
<td>3.63 ± 0.06</td>
<td>1.9 ± 0.04</td>
<td>0.16 ± 0.03</td>
</tr>
</tbody>
</table>

Results for amines, glycerol and myo-inositol are shown.
and kidneys. It was postulated that hyperinsulinaemia which is present in cirrhosis may drive BCAA to the muscle and the kidneys where they are broken down\textsuperscript{28}. Our results do not support this hypothesis particularly in encephalopathy as concentrations of leucine and isoleucine are increased in encephalopathy. If we look at BCAA individually we find that their metabolic fate after the initial transamination and decarboxylation can be very different from one to the other. Leucine is a ketogenic amino acid which can be oxidised to acetyl-CoA. This study provides evidence that ketogenesis is impaired in encephalopathy as is the peripheral utilisation of the ketone bodies and this might explain the increased concentrations of leucine. Valine can only be a gluconeogenic amino acid that could enter the Kreb’s cycle and provide towards the production of ATP. As acetyl-CoA is in short supply Kreb’s cycle can be fuelled from alternative sources such as valine. And this might explain the decreased concentrations of that amino acid.

We do not have an immediate explanation as to why the concentration of isoleucine is high in encephalopathic cirrhotics in our study population. Isoleucine is a ketogenic amino acid and as the production of acetoacetate is increased but its catabolism is not it might be an index of diminished ketogenesis in encephalopathy.

Hyperammonaemia and diminished urea production are well characterised phenomena in cirrhotic patients\textsuperscript{29-31}. Our study showed that cirrhotics had increased levels of glutamate, histidine and arginine and decreased levels of glutamine. This is a pattern which is not in accordance with the previous studies which showed a generalised decrease in those amino acids in chronic liver failure. It is in accordance though with studies in experimental animal models of liver failure. Although other studies in patients suffering acute liver failure confirmed this pattern, our studies in acute liver failure found no differences in any of those substances between patients and controls\textsuperscript{32,33}. Arginine is an amino acid that is an intermediary of the urea cycle. Its observed increased concentrations are in agreement with a decreased urea cycle as is the increased histidine concentrations which is a glutamate precursor.

Glutamine however, was part of the main discriminate function between cirrhotic and encephalopathic patients. Although this might seem paradoxical, there is evidence of increased ammonia production during encephalopathy, which is implicated in its pathogenesis. The fact that glutamine synthesis is impaired may provide another point for the hyperammonaemia of encephalopathy. An alternative pathway to this is the production of amines and TMAO which can assist in the ammonia detoxification. If the findings are confirmed in larger studies and the biomarkers are accurate in differentiating between cirrhosis and pre-encephalitic states they would provide objective non-invasive criteria for the presence of cirrhosis and hepatic encephalopathy.

**Peer-review**

The authors have performed a good study, the manuscript is interesting.

**REFERENCES**


