

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

Konstantinos John Dabos, John Nicholas Plevris, Peter Clive Hayes, Centre of Liver and Digestive Disorders, Royal Infirmary of Edinburgh, Edinburgh EH16 4SA, Scotland, United Kingdom

John Andrew Parkinson, Department of Chemistry, University of Strathclyde, Glasgow G1 1XW, United Kingdom

Ian Howard Sadler, Department of Chemistry, University of Edinburgh, Edinburgh EH16 4SA, Scotland, United Kingdom

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

Author contributions: Plevris JN and Hayes PC conceived the study; Dabos KJ and Plevris JN designed the study; Dabos KJ and Parkinson JA performed the data collection; Sadler IH helped with the data collection; Dabos KJ wrote the manuscript; all authors critically reviewed the manuscript and approved it.

Ethics approval: The study was reviewed and approved by the Lothian Research Ethics Committee Institutional Review Board.

Clinical trial registration: As this trial recruited before 1999 it was not registered with a clinical trials registry.

Informed consent: All study participants or their next of kin provided written informed consent prior to study enrolment.

Conflict-of-interest: The authors do not disclose any conflicts of interest.

Data sharing: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Correspondence to: Konstantinos John Dabos, MD, PhD, Centre of Liver and Digestive Disorders, Royal Infirmary of Edinburgh, 49 Little France Crescent, Edinburgh EH16 4SA, Scotland, United Kingdom. konstantinos.dabos@nhslothian.scot.nhs.uk
Telephone: +44-131-2421627

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 stable 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 acetoacetate (0.23 ± 0.02 vs $0.05 \pm 0.00, P < 0.01$), and b-hydroxybutarate (0.58 ± 0.14 vs $0.08 \pm 0.00, P < 0.01$), lower concentrations of glutamine (0.44 ± 0.08 vs $0.63 \pm 0.03, P < 0.05$), histidine (0.16 ± 0.01 vs $0.36 \pm 0.04, P < 0.01$) and arginine (0.08 ± 0.01 vs $0.14 \pm 0.02, P < 0.03$) and higher concentrations of glutamate (1.36 ± 0.25 vs $0.58 \pm 0.04, P < 0.01$), lactate (1.53 ± 0.11 vs $0.42 \pm 0.05, P < 0.01$), pyruvate (0.11 ± 0.02 vs $0.03 \pm 0.00, P < 0.01$) threonine (0.39 ± 0.02 vs $0.08 \pm 0.01, P < 0.01$) 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 $0.16 \pm 0.02, P < 0.0002$), higher acetoacetate (0.23 ± 0.02 vs $0.41 \pm 0.16, P < 0.05$), leucine (0.33 ± 0.02 vs $0.49 \pm 0.05, P < 0.005$) and isoleucine (0.12 ± 0.02 vs $0.27 \pm 0.02, P < 0.0004$) and lower glutamine (0.44 ± 0.08 vs $0.36 \pm 0.04, P < 0.013$), glycerol (0.53 ± 0.03 vs $0.19 \pm 0.02, P < 0.000$) and myoinositol (0.36 ± 0.04 vs $0.18 \pm 0.02, P < 0.010$) 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 metabonomics can select biomarkers for these diseases.

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

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

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.

nuclear magnetic resonance spectroscopy-based metabonomic study in patients with cirrhosis and hepatic encephalopathy. *World J Hepatol* 2015; 7(12): 1701-1707 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v7/i12/1701.htm> DOI: <http://dx.doi.org/10.4254/wjh.v7.i12.1701>

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 metabonomics, 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]. Metabonomics makes use of multivariate statistical approaches to analyse complex data sets, such as those obtained by ^1H 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 ^1H 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^[8]. 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 D₂O 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 CMPC 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 Lamda 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, b-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 Tukey 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

Table 1 Patients and controls were well matched for age and sex

	Controls	Cirrhosis	Encephalopathy
Age	48.8 ± 9.9	54.3 ± 8.8	56.8 ± 6.0
Sex	M: 10 F: 7	M: 9 F: 9	M: 12 F: 6
CP score	N/A	7.8 ± 1.6	9.9 ± 2.1
Child class a	N/A	1	1
Child class b	N/A	11	6
Child class c	N/A	6	11

Patients with hepatic encephalopathy had more severe liver disease. CP: Child-Pugh; M: Male; F: Female.

Table 3 Results for glycolysis end products and gluconeogenic precursors are shown

	Chemical shift	Cirrhosis	Encephalopathy	Controls
Lactate	1.33	1.53 ± 0.11 ^b	1.41 ± 0.13 ^b	0.42 ± 0.05
Pyruvate	2.38	0.11 ± 0.02 ^b	0.17 ± 0.02 ^b	0.03 ± 0.00
Alanine	1.48	0.77 ± 0.04 ^b	0.73 ± 0.06 ^b	0.61 ± 0.05
Threonine	1.34	0.39 ± 0.02 ^b	0.25 ± 0.01 ^b	0.08 ± 0.1
Glycine	3.57	0.31 ± 0.03 ^b	0.18 ± 0.01 ^b	0.09 ± 0.1
Aspartate	2.82	0.37 ± 0.03 ^b	0.27 ± 0.02 ^b	0.03 ± 0.1

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

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 β-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 controls than encephalopathics (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).

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

	Chemical shift	Cirrhosis	Encephalopathy	Controls
Acetoacetate	2.29	0.23 ± 0.02 ^b	0.41 ± 0.05 ^b	0.05 ± 0.00
B-hydroxybutyr	2.31	0.58 ± 0.14 ^b	0.16 ± 0.02 ^b	0.08 ± 0.00
Leucine	0.96	0.33 ± 0.02	0.49 ± 0.05 ^b	0.35 ± 0.02
Isoleucine	1.01	0.12 ± 0.02	0.27 ± 0.02 ^b	0.13 ± 0.02
Valine	1.04	0.14 ± 0.01 ^d	0.16 ± 0.02 ^d	0.36 ± 0.03
Phenylalanine	7.38	0.08 ± 0.01 ^b	0.06 ± 0.02 ^b	0.02 ± 0.01
Tyrosine	6.91	0.23 ± 0.02 ^b	0.25 ± 0.06 ^b	0.07 ± 0.00
Methionine	2.14	0.07 ± 0.02 ^b	0.08 ± 0.02 ^b	0.03 ± 0.01

Acetoacetate and β-hydroxybutyrate concentrations were significantly higher in patients than controls (^bP < 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 (^dP < 0.01). Leucine was significantly higher if we compared encephalopathics with controls (^bP < 0.01), but there was no difference if we compared cirrhotics and controls. Isoleucine was significantly lower if we compared encephalopathics with controls (^bP < 0.01) but there was no difference between cirrhotics and controls.

Table 4 Results for urea cycle intermediates are shown

	Chemical shift	Cirrhosis	Encephalopathy	Controls
Glutamine	2.46	0.44 ± 0.08 ^b	0.36 ± 0.04 ^b	0.63 ± 0.03
Glutamate	2.36	1.36 ± 0.25 ^d	0.84 ± 0.16 ^a	0.58 ± 0.04
Histidine	7.83	0.16 ± 0.01 ^b	0.18 ± 0.02 ^b	0.36 ± 0.04
Arginine	1.93	0.08 ± 0.01 ^b	0.1 ± 0.01 ^b	0.14 ± 0.02

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

Table 5 Results for amines, glycerol and myo-inositol are shown

	Chemical shift	Cirrhotics	Encephalopathics	Controls
Methylamine	2.54	0.17 ± 0.03	0.19 ± 0.03	0
Dimethylamine	2.72	0.29 ± 0.03	0.31 ± 0.04	0
Tmao	3.27	0.45 ± 0.07	0.51 ± 0.08	0
Glycerol	3.79	0.53 ± 0.09 ^b	0.2 ± 0.02 ^b	0.08 ± 0.02
Myoinositol	3.63	0.37 ± 0.06 ^a	0.19 ± 0.04	0.16 ± 0.03

Methylamine, dimethylamine and TMAO were present in patients and absent in controls. Glycerol concentrations were significantly higher in patients than controls (^b*P* < 0.01). Myo-inositol concentrations were significantly higher in cirrhotics (^a*P* < 0.015) but there were no differences between encephalopathics and controls.

a positive predictive value (PPV) of 100%, a negative predictive value (NPV) of 94% a sensitivity of 95%, a specificity of 100% and an overall accuracy of 98%.

If we compared between patients in groups A and B, β-hydroxybutyrate was significantly lower in encephalopathics (0.58 ± 0.14 vs 0.16 ± 0.02 , *P* < 0.0002). In contrast, acetoacetate was significantly higher in encephalopathics (0.23 ± 0.02 vs 0.41 ± 0.16 , *P* < 0.05). The concentration of 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) were significantly higher in encephalopathics. Glutamine concentrations were lower in encephalopathic patients (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 concentrations (0.36 ± 0.04 vs 0.18 ± 0.02 , *P* < 0.01) were significantly lower in encephalopathic patients.

The strongest segregation was observed with input from β-hydroxybutyrate, glutamine, glycerol and glutamate in the SDA. A discriminant function (sum of concentrations of all four metabolites (β-hydroxybutyrate + glutamine + glycerol + glutamate) in mmols/L < 1.5 for encephalopathics) was created. By performing ROC analysis it had a PPV of 89%, a NPV of 83%, a sensitivity of 84%, a specificity of 88% and an overall accuracy of 87%.

DISCUSSION

Our study has confirmed that significant changes occur in plasma concentrations of amino acids and other key metabolites in patients with cirrhosis in the presence or not of hepatic encephalopathy. By using the metabonomics approach we were able to pinpoint metabolites that could be used to identify a patient with or without cirrhosis and with or without hepatic encephalopathy. We will now look at some particular substances in more detail.

Lactate and the amino acids alanine, threonine, glycine and aspartate are major precursors for gluconeogenesis. Pyruvate is also a central substance in glucose metabolism. In both cirrhotics and encephalopathics we found that the concentrations of those substances 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^[17,18] and animal models^[19,20]. Pyruvate and glycine were part of the discriminant function between cirrhotics and healthy controls.

Our study showed that the concentrations of ketone bodies were significantly increased in both groups of patients compared to controls. In encephalopathics, acetoacetate was even more increased than in cirrhotics 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 acetoacetate is increased in encephalopathics. 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 precarious 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 Krebs 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^[21] and to subsequent studies in animal models that were in accordance with that^[22,23].

Typical changes in plasma amino acid patterns have been found in different studies in patients^[24,25] and experimental animals in chronic liver failure^[26,27]. 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^[24-27] 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 encephalopathics 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

and kidneys. It was postulated that hypersulinaemia which is present in cirrhosis may drive BCAA to the muscle and the kidneys where they are broken down^[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 Krebs cycle and provide towards the production of ATP. As acetyl-CoA is in short supply Krebs 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^[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^[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 in the presence of urea cycle impairment. Glutamate and glutamine were part of the discriminate function between cirrhotics and encephalopathic patients.

In conclusion, 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 and impaired urea cycle. In encephalopathy there is a

reversal in the pattern of BCAA concentrations towards normal. By using SDA 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 were not.

COMMENTS

Background

The irreversible liver damage that characterises liver cirrhosis is a bad prognostic factor. The presence of hepatic encephalopathy, a complication of cirrhosis is considered a further aggravating factor.

Research frontiers

Over the last few years a plethora of studies have looked at non-invasive markers to be used in the detection of cirrhosis. At present, the presence of hepatic encephalopathy can only be assessed clinically. Few studies have approached the metabolic abnormalities of liver cirrhosis and its complication hepatic encephalopathy.

Innovations and breakthroughs

This study provides evidence that a combination of biomarkers can differentiate between healthy volunteers and cirrhotic patients. Another combination of biomarkers can predict the presence of overt hepatic encephalopathy in cirrhotics with remarkable accuracy.

Applications

If the findings are confirmed in larger studies and the biomarkers are accurate in differentiating between cirrhosis and pre-cirrhotic states they would provide objective non-invasive criteria for the presence of cirrhosis and hepatic encephalopathy.

Terminology

¹H-nuclear magnetic resonance spectroscopy is an analytical method that can detect metabolites in small quantities in solutions. It uses a powerful magnetic field and software that can transform magnetic signals and footprints of metabolites into concentrations.

Peer-review

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

REFERENCES

- 1 Garcia-Tsao G, Lim JK. Management and treatment of patients with cirrhosis and portal hypertension: recommendations from the Department of Veterans Affairs Hepatitis C Resource Center Program and the National Hepatitis C Program. *Am J Gastroenterol* 2009; **104**: 1802-1829 [PMID: 19455106 DOI: 10.1038/ajg.2009.360]
- 2 Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol* 2006; **45**: 529-538 [PMID: 16879891 DOI: 10.1016/j.jhep.2006.05.013]
- 3 Bajaj JS. Review article: the modern management of hepatic encephalopathy. *Aliment Pharmacol Ther* 2010; **31**: 537-547 [PMID: 20002027 DOI: 10.1111/j.1365-2036.2009.04211.x]
- 4 Butterworth RF, Giguère JF, Michaud J, Lavoie J, Layrargues GP. Ammonia: key factor in the pathogenesis of hepatic encephalopathy. *Neurochem Pathol* 1987; **6**: 1-12 [PMID: 3306479 DOI: 10.1007/BF02833598]
- 5 Kramer L, Tröbl B, Gendo A, Zauner C, Schneider B, Ferenci P, Madl C. Partial pressure of ammonia versus ammonia in hepatic encephalopathy. *Hepatology* 2000; **31**: 30-34 [PMID: 10613724 DOI: 10.1002/hep.510310107]
- 6 Schmidt LE, Tofteng F, Strauss GI, Larsen FS. Effect of treatment with the Molecular Adsorbents Recirculating System on arterial amino acid levels and cerebral amino acid metabolism in patients with hepatic encephalopathy. *Scand J Gastroenterol* 2004; **39**: 974-980 [PMID: 15513337]
- 7 Plauth M, Egberts EH, Hamster W, Török M, Müller PH, Brand O, Fürst P, Dölle W. Long term treatment of latent portosystemic encephalopathy with branch chain amino acids. A double-blind, placebo-controlled trial. *J Hepatol* 1993; **17**: 308-314 [DOI: 10.1016/0898-2260(93)90033-4]

- 10.1016/S0168-8278(05)80210-7]
- 8 **Jalan R**, Seery JP, Taylor-Robinson SD. Review article: pathogenesis and treatment of chronic hepatic encephalopathy. *Aliment Pharmacol Ther* 1996; **10**: 681-697 [PMID: 8899075]
- 9 **Strauss GI**, Knudsen GM, Kondrup J, Møller K, Larsen FS. Cerebral metabolism of ammonia and amino acids in patients with fulminant hepatic failure. *Gastroenterology* 2001; **121**: 1109-1119 [PMID: 11677203 DOI: 10.1053/gast.2001.29310]
- 10 **Bak LK**, Iversen P, Sørensen M, Keiding S, Vilstrup H, Ott P, Waagepetersen HS, Schousboe A. Metabolic fate of isoleucine in a rat model of hepatic encephalopathy and in cultured neural cells exposed to ammonia. *Metab Brain Dis* 2009; **24**: 135-145 [PMID: 19067142 DOI: 10.1007/s11011-008-9123-4]
- 11 **McCullough AJ**, Glamour T. Differences in amino acid kinetics in cirrhosis. *Gastroenterology* 1993; **104**: 1858-1865 [PMID: 8500747]
- 12 **Jacobsen EB**, Hamberg O, Quistorff B, Ott P. Reduced mitochondrial adenosine triphosphate synthesis in skeletal muscle in patients with Child-Pugh class B and C cirrhosis. *Hepatology* 2001; **34**: 7-12 [PMID: 11431727 DOI: 10.1053/jhep.2001.25451]
- 13 **Nicholson JK**, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 1999; **29**: 1181-1189 [PMID: 10598751 DOI: 10.1080/004982599238047]
- 14 **Gao H**, Lu Q, Liu X, Cong H, Zhao L, Wang H, Lin D. Application of 1H NMR-based metabonomics in the study of metabolic profiling of human hepatocellular carcinoma and liver cirrhosis. *Cancer Sci* 2009; **100**: 782-785 [PMID: 19469021 DOI: 10.1111/j.1349-7006.2009.01086.x]
- 15 **Xue R**, Dong L, Wu H, Liu T, Wang J, Shen X. Gas chromatography/mass spectrometry screening of serum metabolomic biomarkers in hepatitis B virus infected cirrhosis patients. *Clin Chem Lab Med* 2009; **47**: 305-310 [PMID: 19676142 DOI: 10.1515/CCLM.2009.083]
- 16 **Barba I**, Chatauret N, García-Dorado D, Córdoba J. A 1H nuclear magnetic resonance-based metabonomic approach for grading hepatic encephalopathy and monitoring the effects of therapeutic hypothermia in rats. *Liver Int* 2008; **28**: 1141-1148 [PMID: 18637065 DOI: 10.1111/j.1478-3231.2008.01801.x]
- 17 **Feng B**, Wu SM, Lv S, Liu F, Chen HS, Gao Y, Dong FT, Wei L. A novel scoring system for prognostic prediction in d-galactosamine/lipopolysaccharide-induced fulminant hepatic failure BALB/c mice. *BMC Gastroenterol* 2009; **9**: 99 [PMID: 20040118 DOI: 10.1186/1471-230X-9-99]
- 18 **Lindon JC**, Nicholson JK, Everett JR. NMR spectroscopy of biofluids. *Ann Reports NMR Spectroscopy* 1999; **38**: 1-87 [DOI: 10.1016/S0066-4103(08)60035-6]
- 19 **Seglen PO**. Autoregulation of glycolysis, respiration, gluconeogenesis, and glycogen synthesis in isolated parenchymal rat liver cells under aerobic and anaerobic conditions. *Biochim Biophys Acta* 1976; **338**: 317-336 [DOI: 10.1016/0304-4165(74)90292-X]
- 20 **de Blaauw I**, Deutz NE, Boers W, von Meyenfeldt MF. Hepatic amino acid and protein metabolism in non-anorectic, moderately cachectic tumor-bearing rats. *J Hepatol* 1997; **26**: 396-408 [PMID: 9059963]
- 21 **Jalan R**, Wright G, Davies NA, Hodges SJ. L-Ornithine phenylacetate (OP): a novel treatment for hyperammonemia and hepatic encephalopathy. *Med Hypotheses* 2007; **69**: 1064-1069 [PMID: 17467190 DOI: 10.1016/j.mehy.2006.12.061]
- 22 **Davies NA**, Wright G, Ytrebø LM, Stadlbauer V, Fuskevåg OM, Zwingmann C, Davies DC, Habtesion A, Hodges SJ, Jalan R. L-ornithine and phenylacetate synergistically produce sustained reduction in ammonia and brain water in cirrhotic rats. *Hepatology* 2009; **50**: 155-164 [PMID: 19437490 DOI: 10.1002/hep.22897]
- 23 **Ytrebø LM**, Kristiansen RG, Maehre H, Fuskevåg OM, Kalstad T, Revhaug A, Cobos MJ, Jalan R, Rose CF. L-ornithine phenylacetate attenuates increased arterial and extracellular brain ammonia and prevents intracranial hypertension in pigs with acute liver failure. *Hepatology* 2009; **50**: 165-174 [PMID: 19554542]
- 24 **Fischer JE**, Rosen HM, Ebeid AM, James JH, Keane JM, Soeters PB. The effect of normalization of plasma amino acids on hepatic encephalopathy in man. *Surgery* 1976; **80**: 77-91 [PMID: 818729]
- 25 **Plauth M**, Egberts EH, Abele R, Müller PH, Fürst P. Characteristic pattern of free amino acids in plasma and skeletal muscle in stable hepatic cirrhosis. *Hepatogastroenterology* 1990; **37**: 135-139 [PMID: 2312039]
- 26 **Cascino A**, Cangiano C, Fiaccadori F, Ghinelli F, Merli M, Pelosi G, Riggio O, Rossi Fanelli F, Sacchini D, Stortoni M, Capoccacia L. Plasma and cerebrospinal fluid amino acid patterns in hepatic encephalopathy. *Dig Dis Sci* 1982; **27**: 828-832 [PMID: 7105954 DOI: 10.1007/BF01391377]
- 27 **Zwingmann C**, Butterworth R. An update on the role of brain glutamine synthesis and its relation to cell-specific energy metabolism in the hyperammonemic brain: further studies using NMR spectroscopy. *Neurochem Int* 2005; **47**: 19-30 [PMID: 15916833 DOI: 10.1016/j.neuint.2005.04.003]
- 28 **Tessari P**, Biolo G, Inchiostro S, Orlando R, Vettore M, Sergi G. Leucine and phenylalanine kinetics in compensated liver cirrhosis: effects of insulin. *Gastroenterology* 1993; **104**: 1712-1721 [PMID: 8500731]
- 29 **Holmin T**, Agardh CD, Alinder G, Herlin P, Hultberg B. The influence of total hepatectomy on cerebral energy state, ammonia-related amino acids of the brain and plasma amino acids in the rat. *Eur J Clin Invest* 1983; **13**: 215-220 [PMID: 6135613 DOI: 10.1111/j.1365-2362.1983.tb00090.x]
- 30 **Swain MS**, Bergeron M, Audet R, Blei AT, Butterworth RF. Monitoring of neurotransmitter amino acids by means of an indwelling cisterna magna catheter: a comparison of two rodent models of fulminant liver failure. *Hepatology* 1992; **16**: 1028-1035 [PMID: 1356903 DOI: 10.1002/hep.1840160428]
- 31 **Weissenborn K**, Ahl B, Fischer-Wasels D, van den Hoff J, Hecker H, Burchert W, Köstler H. Correlations between magnetic resonance spectroscopy alterations and cerebral ammonia and glucose metabolism in cirrhotic patients with and without hepatic encephalopathy. *Gut* 2007; **56**: 1736-1742 [PMID: 17660226 DOI: 10.1136/gut.2006.110569]
- 32 **Dabos KJ**, Newsome PN, Parkinson JA, Mohammed HH, Sadler IH, Plevris JN, Hayes PC. Biochemical prognostic markers of outcome in non-paracetamol-induced fulminant hepatic failure. *Transplantation* 2004; **77**: 200-205 [PMID: 14742981 DOI: 10.1097/TP.0000100466.22441.37]
- 33 **Dabos KJ**, Newsome PN, Parkinson JA, Davidson JS, Sadler IH, Plevris JN, Hayes PC. A biochemical prognostic model of outcome in paracetamol-induced acute liver injury. *Transplantation* 2005; **80**: 1712-1717 [PMID: 16378066 DOI: 10.1097/TP.0000187879.51616.e0]

P- Reviewer: Frider B, He ST, Pan JJ S- Editor: Tian YL
 L- Editor: A E- Editor: Liu SQ





Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA

Telephone: +1-925-223-8242

Fax: +1-925-223-8243

E-mail: bpgoffice@wjgnet.com

Help Desk: <http://www.wjgnet.com/esps/helpdesk.aspx>
<http://www.wjgnet.com>

