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### Changes in human hepatic metabolism in steatosis and cirrhosis

Schofield, Zoe; Reed, Michelle A.C.; Newsome, Philip; Adams, David; Gunther, Ulrich; Lalor, Patricia

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## Basic Study

**Changes in human hepatic metabolism in steatosis and cirrhosis**

Zoe Schofield, Michelle AC Reed, Philip N Newsome, David H Adams, Ulrich L Günther, Patricia F Lalor

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**Author contributions:** Günther UL and Lalor PF contributed equally to this work, designed the experiments and write the manuscript; Schofield Z performed the experiments and all authors contributed to analysis of data.

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**Abstract****AIM**

To understand the underlying metabolic changes in human liver disease we have applied nuclear magnetic resonance (NMR) metabolomics analysis to human liver tissue.

**METHODS**

We have carried out pilot study using <sup>1</sup>H-NMR to derive metabolomic signatures from human liver from patients with steatosis, nonalcoholic steatohepatitis (NASH) or alcohol-related liver damage (ARLD) to identify species that can predict outcome and discriminate between alcohol and metabolic-induced liver injuries.

**RESULTS**

Changes in branched chain amino acid homeostasis, tricarboxylic acid cycle and purine biosynthesis intermediates along with betaine were associated with the development of cirrhosis in both ARLD and nonalcoholic fatty liver disease. Species such as propylene glycol and as yet unidentified moieties that allowed discrimination

between NASH and ARLD samples were also detected using our approach.

### CONCLUSION

Our high throughput, non-destructive technique for multiple analyte quantification in human liver specimens has potential for identification of biomarkers with prognostic and diagnostic significance.

**Key words:** Human; Liver; Metabolomics; Steatosis; Nuclear magnetic resonance; Alcohol

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**Core tip:** We have for the first time performed a comparative analysis of <sup>1</sup>H-NMR spectra from human liver derived from patients with different, but histologically similar etiologies, and steatotic donor tissue. In agreement with the fibrotic and inflammatory picture in the diseased livers, analytes relating to energy and protein metabolism and ketone body production were altered compared to the donor samples. More importantly, novel combinations of markers that may have diagnostic or prognostic significance were also identified by this approach.

Schofield Z, Reed MAC, Newsome PN, Adams DH, Günther UL, Lalor PF. Changes in human hepatic metabolism in steatosis and cirrhosis. *World J Gastroenterol* 2017; 23(15): 2685-2695 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i15/2685.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i15.2685>

## INTRODUCTION

Rising rates of obesity have led to a dramatic increase in nonalcoholic fatty liver disease (NAFLD), a manifestation of the metabolic syndrome that occurs as a spectrum from fatty liver (steatosis) through inflammation (NASH) to cirrhosis and primary liver cancer. European prevalence of NAFLD is between 15%-44% in adults<sup>[1]</sup> and the prevalence in children is also rising. Although only 10% of patients with steatosis progress to cirrhosis, this still represents a major cause of liver disease, with risk increasing dramatically in patients with type 2 diabetes mellitus. Urgent strategies are required to reduce the burden of fatty liver disease but there are currently no licensed therapies<sup>[2]</sup>, and robust markers that can accurately identify patients at risk of progression from steatosis to NASH are yet to be fully described<sup>[2]</sup>. NASH is histologically indistinguishable from alcohol-induced liver disease but occurs in the absence of significant alcohol consumption<sup>[3]</sup>. Here, development of fatty liver disease in common with cardiovascular disease is driven by poor diet, physical inactivity, insulin resistance and central adiposity but detailed description

of the mechanistic triggers for NAFLD is lacking in part because of the overlap with cardiovascular disease. However it is clear from human studies that the transition from steatosis to steatohepatitis is accompanied by changes in the plasma lipidomic profile<sup>[4]</sup> and that crosstalk between the tricarboxylic acid (TCA) cycle and branched chain amino acids<sup>[5]</sup>, along with oxidative stress in hepatocytes exacerbates the injury.

Many groups have turned to metabolic analysis of diseased livers to identify potential mechanistic drivers or diagnostic markers and to inform future personalized medicine strategies<sup>[6]</sup>. Metabolomics assesses the small molecule composition and biochemical changes in tissue, induced as a consequence of pathogenic processes and has been applied to a variety of human metabolic disease states<sup>[7]</sup>. Studies of human liver disease commonly analyze serological marker profiles, yielding valid evidence for changes in lipid and lipoprotein metabolism<sup>[8]</sup> in the periphery but being limited by potential extrahepatic contribution to the metabolic signature. Rodent models of fatty liver disease are also available permitting hepatic analysis<sup>[7]</sup>, but many require specific genetic manipulations and those which recreate the full spectrum of advanced disease (*e.g.*, presence of fibrosis, inflammation and steatosis) may not reproduce the systemic picture seen in human patients with NASH such as systemic insulin resistance. Thus promising drug candidates identified based upon evidence from rodent models of fatty liver injury have not proven effective in human systems<sup>[2]</sup>. Many metabolomic studies thus far have focused on NAFLD or obesity rather than NASH<sup>[5,9]</sup>, and development of new therapies for NASH is further hampered by the requirement for histological endpoints in clinical trials. We have addressed these issues by performing a metabolomics analysis of human liver tissue with steatosis, NASH and alcohol-related damage (ARLD) by <sup>1</sup>H-NMR. This provides a high throughput, non-destructive and sensitive technique for multiple analyte quantification with an often better reproducibility (and hence greater accuracy) than mass-spectrophotometric studies<sup>[10]</sup> and has the potential to identify species which can discriminate between alcohol-induced and metabolic liver injuries that are hard to separate histologically. Herein we report that an nuclear magnetic resonance (NMR) metabolomics approach allows us to discriminate human metabolic signature changes that accompany development of cirrhosis in both alcoholic and metabolic liver injury. More importantly we have identified key species that permit discrimination between these two etiologies.

## MATERIALS AND METHODS

### Patient liver samples

Human liver samples from patients in the Birmingham liver transplantation programme were used throughout

**Table 1** Patient demographics for samples used for <sup>1</sup>H-NMR analysis of whole liver tissue

Group	Age	Sex	Height (cm)	Weight (kg)	BMI	ALT (IU/L)	AST (IU/L)	Bilirubin ( $\mu$ mol/L)
ARLD	40	F	162	86.7	33	21	-	3
ARLD	49	M	188	104	29.4	33	66	37
ARLD	54	M	179	101	31.5	14	-	135
ARLD	65	F	162	71	27.1	19	-	75
ARLD	49	M	183	66	19.7	43	59	30
mean	52 $\pm$ 9.12		172.75 $\pm$ 12.1	90.68 $\pm$ 17.3	30.25 $\pm$ 5.21	21.75 $\pm$ 11.7	66 $\pm$ 4.94	62.5 $\pm$ 51.1
NASH	60	M	173	110	36.8	29	66	40
NASH	61	M	175	106	34.6	45	126	17
NASH	51	M	178	112	35.3	25	32	30
NASH	51	F	158	57	22.8	12	46	191
NASH	50	F	157	64	26	32	74	145
NASH	49	F	162	86	32.8	54	72	97
NASH	60	M	175	95	31	33	87	90
NASH	52	M	170	113	39.1	37	47	66
NASH	47	M	169	109	38.2	34	51	19
NASH	70	F	164	82	30.5	28	32	13
NASH	44	M	165	101	37.1	36	62	109
NASH	52	M	173	103	34.4	24	37	44
NASH	60	M	179	123	32.1	22	41	27
NASH	61	M	178	97	30.6	18	34	50
mean	55 $\pm$ 7.1		169.7 $\pm$ 7.4	97 $\pm$ 18.9	32.95 $\pm$ 4.6	30.6 $\pm$ 10.8	58 $\pm$ 26.2	67 $\pm$ 53.2
Donor	-	-	-	-	-	-	-	-
Donor	-	-	-	-	-	119	-	14
Donor	-	-	-	-	-	11	-	14
Donor	-	-	-	-	-	-	-	-
Donor	77	M	176	80	25.8	55	-	18
Donor	74	F	-	-	-	-	-	-
Donor	-	-	-	-	-	-	-	-
Donor	-	-	-	-	-	9	-	11
Donor	39	M	176	68.7	22.2	63	126	15
Donor	-	-	-	-	-	-	-	-
Donor	46	M	171	51.3	17.54	30	N/A	23
Donor	-	-	-	-	-	-	-	-
Donor	-	-	-	-	-	-	-	-
Donor	-	-	-	-	-	-	-	-
Donor	-	-	-	-	-	-	-	-
Donor	55	F	163	56.1	21.1	48	97	7

Demographics of our patients at time of transplantation surgery - indicates data unavailable. In particular for most organ donors, constraints of our ethical approval process meant we were unable to access anonymized clinical demographic information from deceased donors. Thus we only have data for selected steatotic donor liver samples. BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate transaminase; NASH: Nonalcoholic steatohepatitis; ARLD: Alcohol-related liver damage.

this study, and all samples were collected with informed written patient consent and local ethics committee approval (06/Q2702/61). Samples were anonymized to ensure no sensitive information was available to the researchers. Material was collected within one hour of removal and fresh liver tissue was either immediately snap frozen in liquid nitrogen for NMR analysis or formalin fixed and embedded in paraffin for histological analysis. We used three types of tissue, firstly normal "Donor" material from healthy livers deemed unsuitable for liver transplantation primarily as a consequence of presence of steatosis. These samples therefore are classed as steatotic. We also used "NASH" or "ARLD" livers collected from patients undergoing liver transplantation. These patients had fulfilled criteria for inclusion onto the transplantation list. Here diagnosis of NASH was defined as presence of histological features of steatosis, lobular inflammation and hepatocyte ballooning in the absence of a history of alcohol excess

(> 14 units/wk) and exclusion of other viral/metabolic or autoimmune etiologies. ARLD patients were defined on the basis of chronic liver damage in the context of excessive alcohol intake in the absence of other viral or autoimmune causes. Tissue was collected from  $n = 16$  donors,  $n = 5$  alcoholic patients and  $n = 14$  NASH patients. Detailed demographic information for donors of explanted cirrhotic liver is supplied in Table 1. Steatotic donor livers rejected for transplantation were used as our steatotic donor liver group. Demographic information for these samples is much more limited as such samples do not come with full biochemical characterisation.

### Histology

Paraffin-embedded samples were cut into 5  $\mu$ m sections and stained using Haematoxylin and Eosin and Van Gieson stains using standard protocols for assessment of tissue morphology, steatosis and fibrosis respectively.



Representative images were captured using a Zeiss AxioScope microscope and Axiovision software.

### Sample preparation for NMR

Tissue samples were stored in a freezer at  $-80^{\circ}\text{C}$  before being prepared for NMR analysis according to previously described methodology<sup>[11]</sup> with the addition of a tissue digestion step using a gentleMACS homogeniser (Miltenyi, United Kingdom). All solvents (HPLC grade Methanol and Chloroform, Sigma Aldrich) were kept on ice at  $-4^{\circ}\text{C}$ . Liver samples (approximately 89 mg or approximately 5 mm<sup>3</sup>) were added to gentleMACS M-Tubes in methanol (8  $\mu\text{L}/\text{mg}$ ) and purified water (2  $\mu\text{L}/\text{mg}$ ) and homogenised using a gentleMACS dissociator. Samples were decanted into 5 mL glass vials. Fresh chloroform (8  $\mu\text{L}/\text{mg}$ ) and water (4  $\mu\text{L}/\text{mg}$ ) were added and samples vortexed for 30 s. The mixture was left to stand on ice before centrifugation (5 min at 2500 rpm followed by another 5 min at 3000 rpm). The polar layer of the resultant multiphasic solution was carefully pipetted into a sterile Eppendorf tube (100  $\mu\text{L}$ ) and air dried using a vacuum concentrator for an hour. All layers were stored at  $-80^{\circ}\text{C}$  for future analysis.

### NMR data collection

All data collection was carried out using a Bruker 600MHz Avance III spectrometer using a 1.7 mm cryoprobe. To acquire 1D <sup>1</sup>H-NMR spectra dried polar metabolites were re-suspended in sodium phosphate buffer in 90% H<sub>2</sub>O and 10% D<sub>2</sub>O (0.1 mol/L, pH 7.09) containing 3-trimethylsilyl-2,2,3,3-d<sub>4</sub>-propionate (TMSP) as an internal chemical shift standard. 1D-<sup>1</sup>H-NOESY spectra were collected at 298 K with a 12 ppm spectral width, 32k data points and 256 scans.

### NMR data analysis

Spectra were processed using NMRlab and Metabolab programmes<sup>[12,13]</sup> within Matlab, version R2015b (MathWorks, Massachusetts, United States). Briefly, the first data point was multiplied by 0.5, a line-broadening window function was applied and the data were zero-filled to 32768 points prior to Fourier transformation. All spectra were rephased manually after initial automated phase correction. Before analysis of the spectra, all spectra were aligned on TMSP, a spline baseline correction was applied, the water and TMSP regions were excluded and the total spectra area (TSA) of each spectrum was scaled to 1. However, scaling to the TMSP signal produced almost identical results as total spectra area scaling.

Resonances were assigned using Chenomx 8.1 (Alberta, Canada, 2015). Calibration was carried out manually using alanine as a reference peak ( $\delta = 1.46$  ppm). The human metabolome database (HMDB)<sup>[14]</sup> was used to identify metabolites not covered by Chenomx.

### Statistical analysis

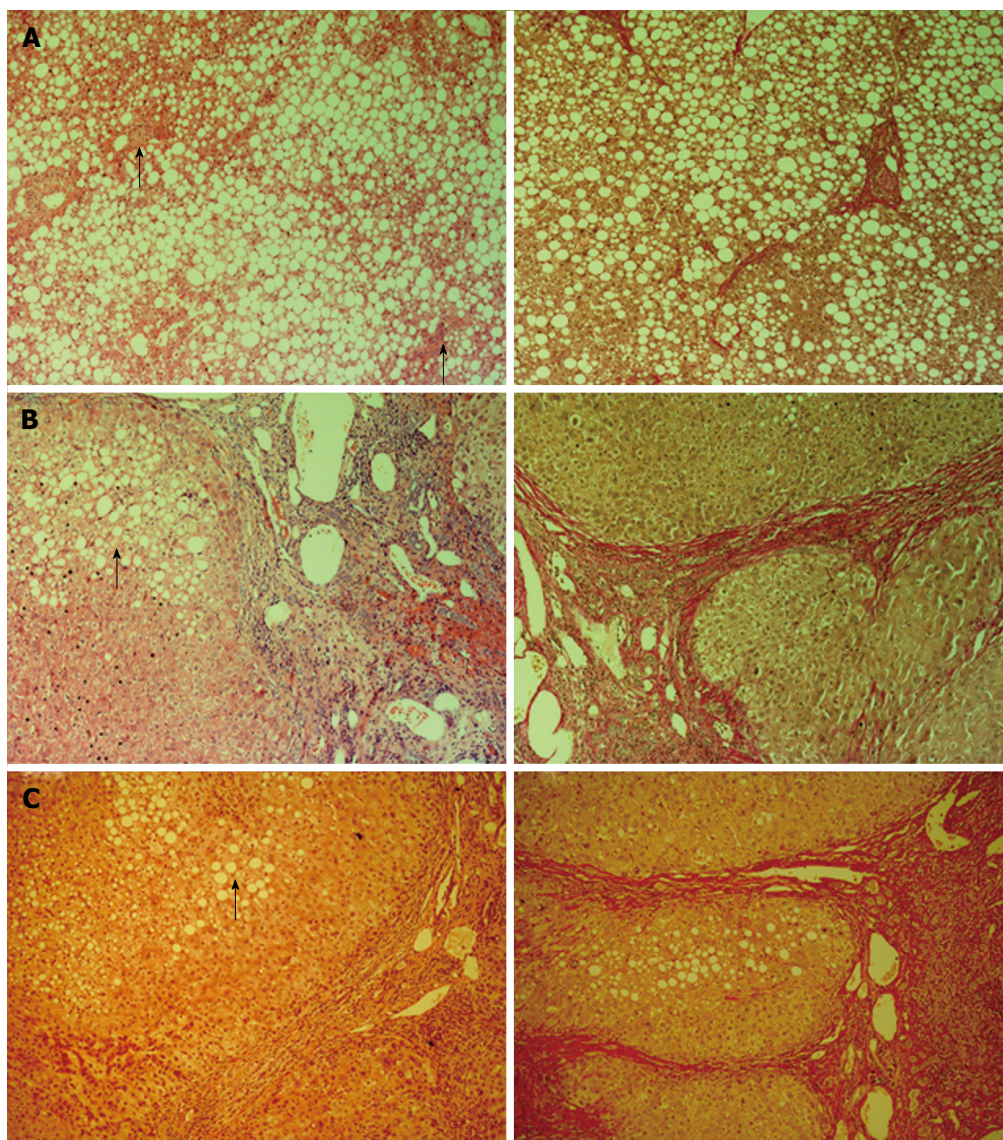
In normalised <sup>1</sup>H NMR spectra, one well-resolved peak

was picked for each metabolite in the first spectrum and peaks were picked in the other spectra in an automated manner using in-house subroutines of MetaboLab. Three classes were defined: donor (9 spectra), NASH (6) and ARLD (5). The mean and standard deviations for each class were calculated. For each metabolite, the unpaired *t*-test (Welch's *t*-test) was used (using *ttest2u* from the PLSTOOLBOX for Matlab by Eigenvector Research Inc, Washington, United States) with a 5% cut-off (*P* value < 0.05) to test the null hypothesis that the relative peak intensities for pairs of the different classes have the same mean, variances not assumed to be equal.

## RESULTS

In order to determine differences in metabolic signatures in liver tissue during NASH, we compared NMR profiles with those from "donor" steatotic liver and importantly from tissue collected from patients with ARLD, which is histologically indistinguishable from NASH<sup>[3,15]</sup>. The donor tissue samples all showed extensive macrovesicular steatosis throughout the lobule (Figure 1A), and some cases exhibited areas of localised inflammation (see arrows Figure 1A). Table 1 shows the available biochemical and demographic data for our patient groups and confirms that the patients in the ARLD and NASH groups were age matched ( $52 \pm 9.12$  years vs  $55 \pm 7.16$  years respectively) and their BMI was not significantly different at time of transplant ( $30.25 \pm 5.21$  vs  $32.95 \pm 4.6$  respectively, Table 1). Both diseased groups had extensive bridging fibrosis and inflammatory infiltrate in the liver (Figure 1B and C). Areas of steatosis were occasionally noted in the NASH and ARLD samples (see arrows Figure 1B and C) but were not present in all livers. Given the extensive fibrosis and resistant texture of our liver specimens we were keen to confirm the reproducibility of our extraction technique on tougher, cirrhotic specimens compared to the softer donor tissue. Figure 2 shows representative 1D <sup>1</sup>H-NMR spectra from our samples and illustrates that the polar extracts such as the branched chain amino acids were extremely consistent within each of the three groups, yielding a clear separation for a panel of metabolites between donor and NASH groups, albeit with different concentrations of species present between tissue types. Reproducibility of our analytical workflow was confirmed by performing independent repeat analysis using the same patient samples on separate occasions (see Supplemental Figure 2).

Key findings from the 1D proton-NMR are summarised in Figure 3 and confirm that many key metabolites were significantly different between the patient groups (see analysis in Supplemental Table 1). In agreement with the histological picture of steatosis in the donor samples and a more fibrotic and inflammatory picture in the NASH livers, analytes

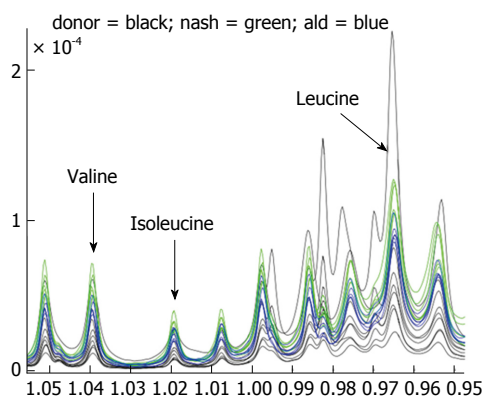


**Figure 1** Histological staining of human liver tissue. Representative images of donor tissue (A), NASH tissue (B) and ARLD liver (C) stained using haematoxylin and eosin (left panel) or Van Gieson stain (right panel). Bar = 100  $\mu$ m and images were captured at 10  $\times$  original magnification. Data are representative of 6-14 samples in each group. Arrows in A indicate areas of localised inflammation present in our steatotic donor livers and arrows in B and C show steatotic hepatocytes. NASH: Nonalcoholic steatohepatitis; ARLD: Alcohol-related liver damage.

relating to energy and protein metabolism and ketone body production were altered compared to the donor samples. Whilst many metabolites were similarly altered in the NASH and ARLD cohorts compared to donor livers, there were key differences that discriminated NASH from ARLD livers. We were able to identify approximately 60 metabolites in NMR spectra of polar extracts, of which 16 were significantly altered between the groups, one of which could not be identified. The branched chain alpha amino acids leucine, valine and isoleucine were all increased in concentration in cirrhotic livers (NASH and ARLD) compared to donor tissue, but interestingly concentrations of alanine, glutamate and glycine were comparable in all livers. The amino acid betaine was particularly abundant in the alcoholic and NASH livers compared to steatotic samples. Other interme-

diates in the TCA cycle, succinate and fumarate were differentially regulated in disease with no change in fumarate levels across groups, but in contrast a significant elevation in succinate levels associated with both alcohol induced liver injury and NASH (Figure 3). As expected we found significant elevations in hepatic glucose concentrations in both NASH and ARLD, and we also noted significant elevations in hepatic lactate during cirrhosis, particularly in the NASH liver specimens. A modest but non-significant elevation in uracil levels present in cirrhosis was accompanied by a modest decrease in uridine levels. The common food additive propylene glycol was also increased in both cirrhotic groups and particularly the alcohol group, a pattern also evident for formate concentrations that were highest in alcoholic liver disease. Finally, an as yet unidentified metabolite with a signal at 7.685 ppm





**Figure 2 Representative  $^1\text{H-NMR}$  spectra.** Representative fraction intensity traces from the branched chain amino acid region of the  $^1\text{H-NMR}$  data for normal (black) NASH (green) and ARLD (blue) livers showing consistency of separation between groups. NASH: Nonalcoholic steatohepatitis; ARLD: Alcohol-related liver damage.

was particularly valuable for differential comparisons between the cirrhotic samples with high levels in NASH livers and overlap with the donor specimens, but concentrations in ARLD being somewhat lower than the steatotic donors (Supplemental Figure 1). This contrasts with two other unidentified metabolites at 5.41 and 5.43 ppm that were significantly lower in ARLD livers.

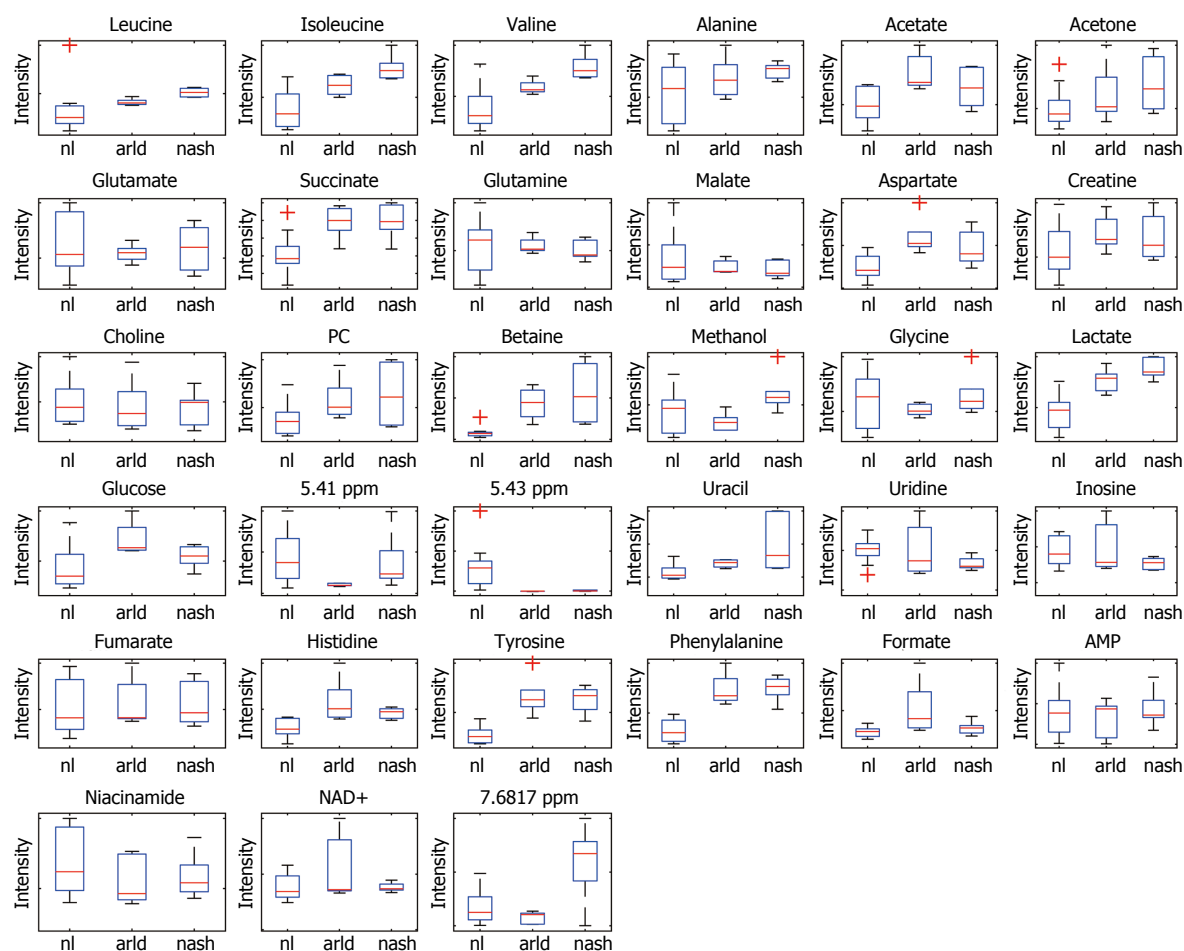
We also compared metabolite levels between ARLD and NASH liver samples in comparison to steatotic livers (Figure 4). This analysis showed that the elevations in amino acids valine and isoleucine in NASH were more pronounced than seen in the ARLD specimens, and also showed the raised formate and glucose in alcoholic livers. However the most impressive differences were observed in two yet unidentified metabolites. We observed significant decreases in all three metabolites which were much reduced in concentration in ARLD livers with the metabolite at 5.43 also significantly reduced in NASH compared to the other patient groups. Again the peak at 7.68 ppm was significantly elevated in NASH livers relative to normal whilst it was reduced in ARLD livers. We also performed a correlation analysis on a small subsection of our cirrhotic patients from whom we had good biochemical data. Analysis of these 10 patients is shown in Figure 5 and shows that accumulation of analytes linked to impaired liver function such as glutamate and lactate, correlated with bilirubin concentration at time of transplant. The same was true for unknown metabolite 7.68 ppm. We also saw a trend for decreased hepatic aspartate levels with age.

## DISCUSSION

We used an NMR metabolomics analysis to generate information on the metabolic picture of human liver tissue in the context of simple steatosis alone or in more advanced disease associated with both fibrosis and hepatic inflammation. NMR was chosen as the

analytical tool for this study by virtue of its ability to rapidly and simply process small volumes of sample to yield unbiased, non-selective structural information on our analytes. Insulin resistance<sup>[16]</sup> and hyperglycaemia are characteristic of NAFLD<sup>[17]</sup> with elevated serum glucose lactate, and glutamate/glutamine levels characterising different stages in the progression to NASH<sup>[18]</sup>. One gas chromatography study has compared the lipid profile of steatotic and NASH livers<sup>[4]</sup> and highlighted changes in lipid and fatty acid homeostasis that accompany NAFLD and are echoed in our own results. We have confirmed evidence from several studies suggesting that glucose homeostasis and branched chain amino acid concentrations are modified in the liver and serum in NAFLD<sup>[9,17-19]</sup>. Previous NMR analysis of serum from patients with cirrhosis confirms significant changes in plasma amino acid concentrations, in particular in patients with encephalopathy<sup>[20]</sup>. We observed elevated hepatic concentrations of the alpha amino acids leucine, valine and isoleucine in accordance with the reported abnormal regulation of hepatic amino acid metabolism in cirrhosis<sup>[21,22]</sup> and lower BCAA oxidation observed in alcohol injury and cirrhosis<sup>[21]</sup>. Interestingly in liver disease one might expect characteristically low serum levels of some BCAA due to changes in protein and amino acid metabolism<sup>[23]</sup>. Diminished release of leucine from muscle is common<sup>[24]</sup> and studies suggest that improved glucose sensitivity in the fed state during cirrhosis may suppress leucine oxidation in the periphery<sup>[25]</sup>. Indeed valine supplementation has been suggested to be beneficial in reduction of fibrosis in rodent models of liver cirrhosis<sup>[26]</sup>, although administration to patients with cirrhosis results in higher serum levels than for control subjects<sup>[27]</sup>.

Other amino acids were differentially regulated however, with glutamate unchanged in NASH and ARLD compared to steatotic livers. Previous data from NMR analysis of obese patients undergoing bariatric surgery has suggested that glutamate levels in the liver reduce as the severity of steatosis increases<sup>[9]</sup> whilst others, in agreement with our results, reported that liver levels are unchanged if steatosis is compared to NASH<sup>[17]</sup>. Thus it is likely that levels in all our patient groups vary compared to healthy liver, in part due to increased utilisation for glutathione generation, particularly in the context of endotoxemia which may accompany cirrhosis<sup>[28]</sup>. This may also fit with the reported reductions in serum glutamate seen in ARLD-related cirrhosis<sup>[29]</sup>. Importantly, some of our patients are given personalized nutritional support that can include both vitamin supplementation (Thiamine and Vitamin B strong) as well as high energy protein supplements such as "Fortisips" and "Forticreme" or "Ensure". However these are not given to all patients and are most often used for those with significant sarcopenia and reduction in BMI. Thus we do not supplement in a manner that would explain changes in BCAA metabolism in all patients. Glycine is involved in



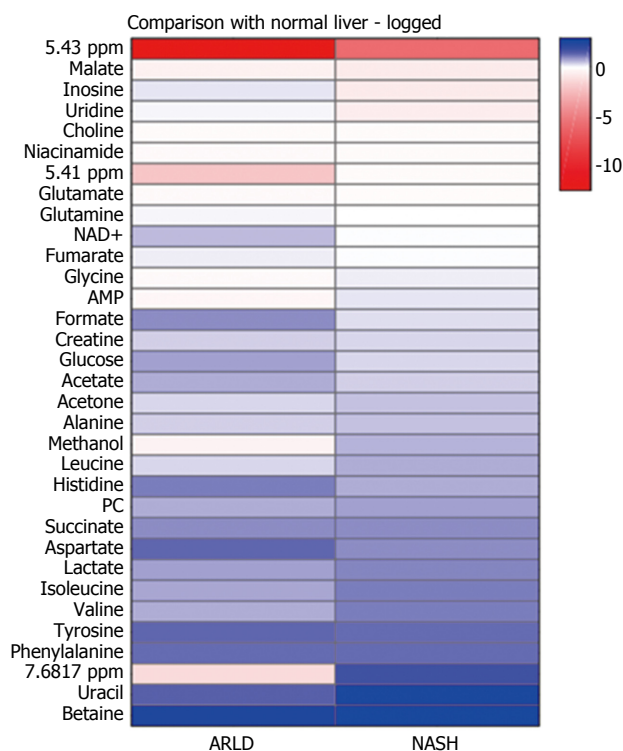
**Figure 3** Liver disease is associated with significant changes in energy and protein metabolism and ketone body production. Fraction intensity boxplots for indicated metabolites in normal liver (nl), ARLD (arld) and NASH (nash) liver samples. For each metabolite shown, an unpaired *t*-test (Welch's *t*-test) was calculated with a 5% cut-off to test the null hypothesis that the relative peak intensities for pairs of the different classes have the same mean, variances not assumed to be equal. The solid line indicates the median fractional intensity, and the box shows the interquartile range. Outlier samples are indicated by red crosses and statistical analyses are indicated in Supplemental Table 1. NASH: Nonalcoholic steatohepatitis; ARLD: Alcohol-related liver damage.

purine biosynthesis and collagen synthesis within the liver, so it is interesting to note the significant decrease in hepatic concentration, particularly in alcoholic liver disease where we see extensive fibrosis. Glycine is also an important component in one carbon metabolism and involved in the generation of *S*-adenosylmethionine (SAMe), a major methyl donor produced and consumed in the liver. The glycine methyltransferase enzyme is reportedly reduced in cirrhotic liver<sup>[30]</sup> and mice lacking this enzyme develop steatosis that progresses to steatohepatitis and cirrhosis<sup>[30]</sup>. Glycine also plays a role in liver regeneration<sup>[31]</sup> thereby confirming the important protective role of SAMe, conforming with our report of reduced levels in human cirrhotic liver and highlighting how reduced glycine levels can exacerbate injury.

Our data showing dramatic increases in hepatic betaine levels in ARLD and NASH are also important in this context. Betaine is another metabolite previously linked to cirrhosis with serological levels having some merit in identification of HCC vs non-malignant liver conditions<sup>[32]</sup>. It is a breakdown product of choline and acts as a methyl donor in the generation of methionine

and can reduce hepatic fat accumulation through inhibition of PPAR- $\alpha$ <sup>[33]</sup>, and maintain SAMe levels. Early animal studies suggested there may be an anti-steatotic benefit from dietary supplementation<sup>[34]</sup> leading to trials of betaine as a treatment strategy in NASH with some reports of histological and biochemical improvement after a year of therapy. However this initial data was not confirmed in larger, better designed studies<sup>[35]</sup> and it may be that once steatosis has progressed to fibrosis and steatohepatitis, levels of betaine increase in the liver as a protective mechanism and this overrides any additional benefit from dietary supplementation.

We also report that the TCA cycle intermediates, succinate and fumarate were differentially regulated in disease with no change in fumarate levels across groups, but in contrast a significant elevation in succinate levels associated with both alcohol induced liver injury and NASH. Plasma levels of succinate have been reported to increase in murine models of liver injury including administration of methionine choline-deficient diet and coupled with reduced fecal



**Figure 4** Heat map comparing the alcohol-related liver damage and nonalcoholic steatohepatitis spectra with the donor spectra. The squares are coloured according to: log2 (mean fractional intensity of metabolite X in class Y / mean fractional intensity of metabolite X in donor class) with blue indicating that metabolite X is higher in the class Y and red indicating that metabolite X is lower in the class Y. NASH: Nonalcoholic steatohepatitis; ARLD: Alcohol-related liver damage.

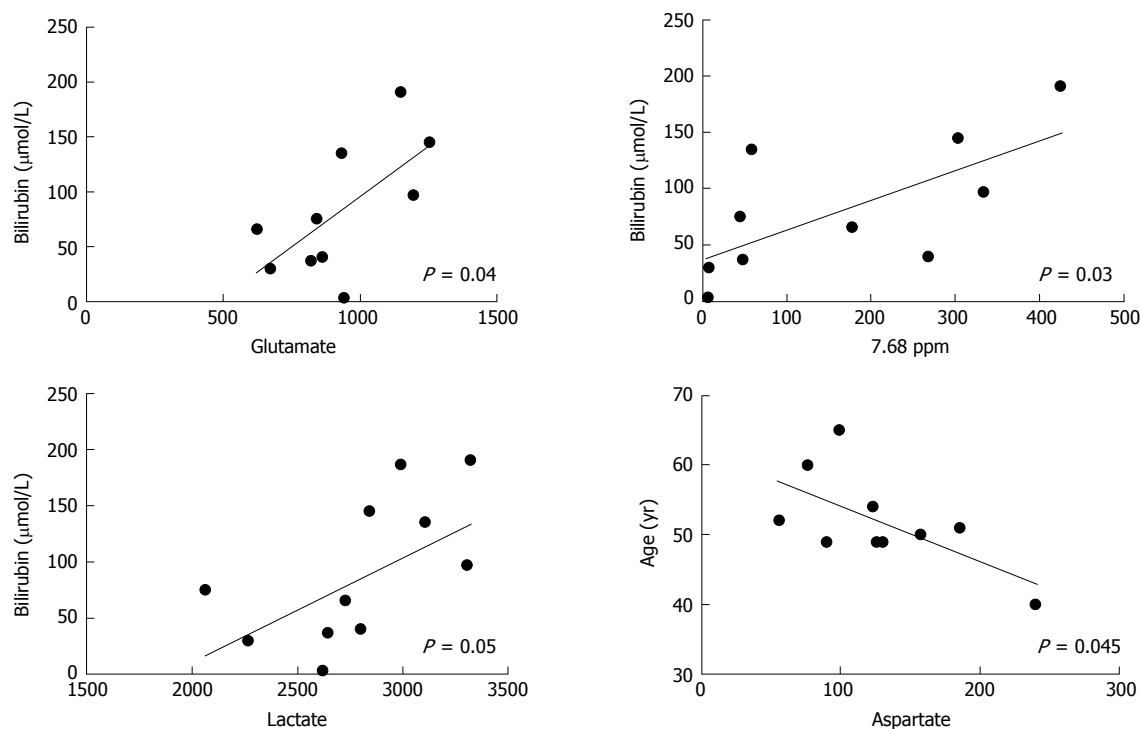
excretion of succinate in insulin-resistant rats<sup>[7]</sup>. Increased succinate suggests malfunction of succinate dehydrogenase. Others also confirm that succinate levels are elevated in cirrhotic liver tissue compared to normal livers<sup>[36]</sup>. Importantly succinate is involved in profibrotic signalling to hepatic stellate cells<sup>[37]</sup> and so our reported hepatic elevations in the context of fibrogenesis in the ARLD and NASH samples fits with this picture. Other metabolites also reiterate the dysregulation of hepatic carbohydrate metabolism that characterises NASH. For example, lactate is used in the Cori cycle for synthesis of glucose, and taken up by healthy liver for this purpose. Reduced hepatic gluconeogenesis and accelerated glycolysis in the splanchnic region in acute liver failure lead to release of lactate. Lactate is also increased in cirrhosis where fasting levels correlate with portal pressure<sup>[38]</sup>. Furthermore a correlation between systemic lactate levels and bilirubin has been observed during living-donor liver transplantation<sup>[39]</sup> and linked to liver function. This is in agreement with our elevated concentrations of lactate in NASH and ARLD and reduced gluconeogenesis in the context of cirrhosis, and our noted correlation between lactate and bilirubin levels. Interestingly the ability of hepatocytes to utilise lactate as fuel for gluconeogenesis has been reported to decrease with age<sup>[40]</sup> due to gradual impairment of mitochondrial

function. Similarly we may be observing age related change in mitochondrial operation of the malate-aspartate shuttle<sup>[41,42]</sup> and reduced export of aspartate which could explain our negative correlation between aspartate levels and age. Thus we may be observing increased utility of aspartate as fuel for gluconeogenesis in our older livers.

An intrinsic link between pyrimidine metabolism and fatty liver disease has been reported previously<sup>[43]</sup> with uridine having protective effects within the liver linked to mitochondrial respiration<sup>[43]</sup>. Excessive consumption or uridine as a protective mechanism in cirrhosis could explain our noted reduction in concentration in the chronically diseased livers. It is however also possible that pyrimidine synthesis is impaired. This might be consistent with increasing succinate levels, and accumulation of lactate, which may reflect a dysfunctional TCA cycle. Certainly disruptions in TCA cycle function have been reported in cirrhosis, linked to reduced intracellular ATP<sup>[44]</sup>. Formate is also made within hepatocytes during the folate cycle and used for pyrimidine synthesis. ARLD is often associated with a deficiency in folate, partly through reduced dietary intake in many alcoholics but also as a consequence of reduced liver uptake and urinary excretion<sup>[45]</sup>. This makes our increased intrahepatic formate levels harder to explain, but may suggest accumulation as a consequence of impaired pyrimidine synthesis in cirrhosis. Certainly in reduced folate situations, mitochondrial formate transport decreases, as does purine synthesis<sup>[46]</sup>.

Whilst many of the metabolic changes discussed above have been reported in other studies, there are several interesting, novel observations in our analysis. Firstly we noted significant increases in hepatic propyleneglycol in both ARLD and NASH. This is a food additive often added to medicinal formulations such as paracetamol as a solubilizing agent<sup>[47]</sup>. Thus increased levels may simply relate to pain relief or other medications taken by our cirrhotic patients. However it is interesting to note that propyleneglycol has been suggested to inhibit the function of CYP4502E1<sup>[48]</sup>, and in this respect the notable increase in ARLD livers may be mechanistically significant. More importantly in the context of NASH, studies have suggested that dietary supplementation may be beneficial for the treatment of insulin resistance<sup>[49]</sup> and that glucose tolerance and skeletal muscle GLUT-4 translocation may improve after treatment. Interestingly, we have also identified high levels of an unidentified aromatic compound at 7.685 ppm in NASH but not in ARLD samples. Use of this metabolite either alone or in combination with BCAAs levels, would clearly discriminate between NASH and ARLD. This could potentially be a drug metabolite since patients with NASH are likely to be taking one or a combination of drugs to manage their metabolic syndrome (including Metformin, GLP-1 analogues, Glycazide, insulin, statins and blood pressure medications). The placement





**Figure 5 Correlation analysis illustrating relationship between key metabolites and demographic parameters in patients with cirrhosis.** Data are from a smaller cohort of patients with cirrhosis relating to ARLD or NASH for whom our full demographic data was available. Dots indicate individual patient data ( $n = 10$ ) and analyses shown are those with significant correlation ( $\alpha < 0.05$ ) for fraction intensity of metabolites vs bilirubin concentration or age. NASH: Nonalcoholic steatohepatitis; ARLD: Alcohol-related liver damage.

of the peak suggests that it is unlikely to represent metformin. However the metabolite was found in all NASH samples and there is no drug that would have been administered to all patients. Also some patients with ARLD may be given drugs to treat diabetic complications, but the metabolite was not present in any of these samples. A similarly good discrimination between NASH and ARLD would be possible based on unidentified metabolites at 5.41 and 5.43 ppm. These signals could potentially represent sugar species that increase in ARLD but not NASH. Further studies based on a larger number of samples need to be performed to identify these metabolites and to clarify whether they represent endogenous metabolites or represent a different metabolism pattern for a therapeutic compound administered to pre-transplant patients. Similarly, future focussed serological analysis directed at the unknown species we have identified will aid assessment of potential diagnostic value. This is important as we would wish to develop diagnostic markers that could ultimately be measured in blood samples rather than tissue specimens. Recent evidence from patients with metabolic disturbance associated with polycystic ovary syndrome confirms that it is indeed possible to detect changes in circulating amino acids and carbohydrates in serum using an NMR approach<sup>[50]</sup>. In addition, given the important contribution of exercise to management of NAFLD<sup>[51]</sup> and links between skeletal muscle activity and hepatic metabolism<sup>[52,53]</sup> it would be important to assess the contribution of sarcopenia in advanced cirrhosis to the

hepatic metabolome.

In conclusion, we have for the first time performed a comparative pilot study of <sup>1</sup>H-NMR spectra from human liver derived from patients with different, but histologically similar etiologies and steatotic donor tissue. In agreement with the fibrotic and inflammatory picture in the diseased livers, analytes relating to energy and protein metabolism and ketone body production were altered compared to the donor samples. More importantly, novel combinations of markers that may have diagnostic or prognostic significance were also identified.

## COMMENTS

### Background

Robust markers that can accurately identify patients at risk of progression from steatosis to nonalcoholic steatohepatitis are lacking. To understand the underlying metabolic changes in human liver disease we have applied nuclear magnetic resonance (NMR) metabolomics analysis to human liver tissue.

### Research frontiers

Metabolic analysis of human livers has the potential to identify mechanistic drivers or diagnostic markers of disease and thus to inform future personalized medicine strategies.

### Innovations and breakthroughs

The authors have for the first time performed a comparative analysis of <sup>1</sup>H-NMR spectra from human liver derived from patients with different, but histologically similar etiologies, and steatotic donor tissue. Importantly, novel combinations of markers that may have diagnostic or prognostic significance were identified by this approach.

## Applications

The methodology that the authors have developed for generating NMR spectra from human liver tissue, and the molecular characterisation they have performed will be of interest to scientists and clinicians studying hepatic metabolism. Novel markers they may have prognostic potential.

## Terminology

NMR - An analytical technique based on the magnetic properties of specific atomic nuclei that is used to determine the identity and structure of complex molecules.

## Peer-review

This is an interesting study on an interesting topic. Recent results indicate that mitochondrial UCP3 activity affects metabolism well beyond fatty acid oxidation, regulating biochemical pathways associated with amino acid metabolism and redox status.

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