Research Article

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Cirrhosis

Multivariate metabotyping of plasma predicts survival in patients with decompensated cirrhosis

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Background & Aims: Predicting survival in decompensated cirrhosis (DC) is important in decision making for liver transplantation and resource allocation. We investigated whether high-resolution metabolic profiling can determine a metabolic phenotype associated with 90-day survival.

Methods: Two hundred and forty-eight subjects underwent plasma metabotyping by ^{1}H nuclear magnetic resonance (NMR) spectroscopy and reversed-phase ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry (UPLC-TOF-MS; DC: 80-derivation set, 101-validation; stable cirrhosis (CLD) 20 and 47 healthy controls (HC)).

Results: ^{1}H NMR metabotyping accurately discriminated between surviving and non-surviving patients with DC. The NMR plasma profiles of non-survivors were attributed to reduced phosphatidylcholines and lipid resonances, with increased lactate, tyrosine, methionine and phenylalanine signal intensities. This was confirmed on external validation (area under the receiver operating curve [AUROC] = 0.96 (95% CI 0.90–1.00, sensitivity 98%, specificity 89%). UPLC-TOF-MS confirmed that lysophosphatidylcholines and phosphatidylcholines [LPC/PC] were downregulated in non-survivors (UPLC-TOF-MS profiles AUROC of 0.94 (95% CI 0.89–0.98, sensitivity 100%, specificity 85% [positive ion detection])). LPC concentrations negatively correlated with circulating markers of cell death (M30 and M65) levels in DC. Histological examination of liver tissue from DC patients confirmed increased hepatocyte cell death compared to controls. Cross liver sampling at time of liver transplantation demonstrated that hepatic endothelial beds are a source of increased circulating total cytokeratin-18 in DC.

Conclusion: Plasma metabotyping accurately predicts mortality in DC. LPC and amino acid dysregulation is associated with increased mortality and severity of disease reflecting hepatocyte cell death.

Keywords: Metabonomics; Metabolomics; Metabolic profiling; Personalised medicine; Outcome prediction; Acute on chronic liver failure.

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Abbreviations: ALF, acute liver failure; ACLF, acute on chronic liver failure; CLIF-SOFA, chronic liver failure sequential organ failure assessment; CPS, Child-Pugh Score; CPMG, Carr-Purcell-Meiboom-Gill; CV-ANOVA, cross-validated analysis of variance; ESI, electrospray ionisation; GC-MS, gas chromatography mass spectrometry; INR, international normalised ratio; LPC, lysophosphatidylcholine; MELD, model for end-stage liver disease; MHE, minimal hepatic encephalopathy; MS, mass spectrometry; NMR, nuclear magnetic resonance; PC, phosphocholine; PCA, principal components analysis; PLSDA, partial least squares discriminant analysis; OPLS, orthogonal projection least squares; STOCSY, statistical correlation spectroscopy; TOF, time-of-flight; UPLC, ultra-performance liquid chromatography; UKELD, United Kingdom end-stage liver disease.

Introduction

The global incidence of cirrhosis is rising rapidly, owing to an increased prevalence of alcohol-related liver disease, non-alcoholic fatty liver disease, and viral hepatitis [1]. Patients with cirrhosis are prone to decompensation, requiring hospital treatment and can progress to acute on chronic liver failure (ACLF) [2], which requires admission to intensive care with an associated high short-term mortality and significant economic cost [3].

Several methods of outcome prediction in cirrhosis are currently used. The Model for End-Stage Liver Disease (MELD) is the most commonly applied and is used for listing and prioritisation in liver transplantation throughout the world. Despite the success of MELD, several limitations exist concerning the

reproducibility of prothrombin time measurement and the limitations of creatinine [4] as a marker of renal function in patients with cirrhosis. The performance of MELD for outcome prediction is best in patients with stable cirrhosis, but is less accurate for patients with acute on chronic liver failure (ACLF) [5]. Therefore organ failure based scores such as the recently developed CLIF-SOFA (chronic liver failure sequential organ failure assessment) score have been developed [2,6].

Exploratory metabolic profiling or metabotyping involves untargeted measurements of low molecular weight (<1 kDa) compounds using nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) in biofluids or tissues [7]. The response of complex spectral data is then assessed using multivariate statistical techniques [8] to determine which metabolites or metabolite combinations most accurately describe differences seen between classes (control or diseased cohorts or a state pre- or post-intervention (e.g. drug treatment)).

Since the metabolic profile is typically comprised of hundreds or thousands of signals depending on the technique, it is, potentially, a highly valuable methodology in delivering personalised healthcare [9], namely a highly discriminant prediction of response or diagnostic accuracy. The use of pre-interventional phenotypes to predict the outcome of intervention based on mathematical modelling is termed "pharmacometabonomics" [10] and the approach has been widely used to predict drug metabolism [11] and outcomes of cancer therapy, toxicity [12] and safety.

Metabolic profiling has been applied in inflammatory bowel disease [13,14], hepatocellular carcinoma [15,16] and to a limited extent, liver failure [17]. The metabolites detected by NMR reflect a number of roles of the liver in glucose, lipid, amino acid and urea metabolism and these have been investigated in acute liver failure (ALF) by proof-of-principle measurements [17,18] and metabolic phenotypes specific to patients with minimal hepatic encephalopathy (MHE) [19] or high MELD score [20].

Liver failure secondary to hepatitis B has been characterised using MS in tandem with either gas chromatography (GC-MS) or liquid chromatography (LC-MS) [21]. Decreases in plasma glycerophosphocholine and phosphatidylcholine (PC) levels occur [22], and are common markers between different aetiologies of cirrhosis [23,24]. Apoptosis of hepatic endothelial beds may be responsible for this lipid dysregulation. Higher levels of circulating cell death markers have been demonstrated in ALF [25,26], and recently in decompensated cirrhosis (DC) and ACLF [27]. No studies have assessed how profiling could prognosticate and none uses a combination of technologies to develop a global overview of the metabolic signature of poor survival in DC.

In this study, we metabotype plasma in patients with DC to: 1) determine and validate a ^{1}H NMR metabotype of 90-day mortality, which we hypothesise could be more accurate than MELD or CLIF-SOFA; and 2) characterise the lipids of the ^{1}H NMR profile by UPLC-TOF-MS.

Materials and methods

Patients and sample collection

Between December 2008 and January 2011, 80 patients with DC referred to the Institute of Liver Studies, Kings College Hospital, London, were recruited and followed for 90 days. Cirrhosis was defined by at least two compatible diagnostic tests from the following: liver biopsy (fibrosis grade 5 or more), radiologic (ultrasound, computed tomography or magnetic resonance imaging), clinical (presence of hepatocellular jaundice/ascites/hepatic encephalopathy [HE]) or biochemical (hyperbilirubinaemia, prolonged prothrombin time and/or thrombocytopaenia) to provide evidence of cirrhosis. Outcome was defined as either spontaneous survival or death/transplantation. Here, we define decompensation as an acute episode of variceal bleeding, jaundice, encephalopathy, ascites, sepsis or renal dysfunction requiring hospital admission. The study was approved by the local ethics committee (#08/H0702/74) and patients, or their nominee gave written, informed consent within 48 h of presentation. Exclusion criteria were presence of hepatocellular carcinoma, non-hepatic malignancy, non-cirrhotic portal hypertension, chronic liver disease but not cirrhotic and acute liver failure. Liver intensive care unit admission was offered to patients if deemed appropriate by the referring hepatologist and/or intensivist. All patients were managed following standard evidence-based protocols by a specialist multidisciplinary team [28,29].

Blood was drawn within 24 h of admission to hospital into lithium heparin-containing vacuum tubes (BD Vacutainer, BD, Franklin Lakes, NJ, USA) and centrifuged at 12,000 g for 10 min within 1 h of sample collection. Plasma aliquots were stored immediately at −80 °C until further analysis. Data collected at time of sample collection were age, gender, aetiology of cirrhosis, past medical history, medication use, dietary history and 72 h dietary recall, alcohol and recreational drug use, recent exercise history, bedside physiology and blood biochemistry. The Child-Pugh Score (CPS) [30], MELD [31], United Kingdom end-stage liver disease (UKELD) [32], CLIF-SOFA [2], CLIF AD [33] and CLIF-C ACLF [6] scores were calculated from data taken on the same day as blood was drawn for metabolic profiling. Twenty patients with stable cirrhosis and 20 age- and sex-matched healthy controls (HC) with no history of liver disease, excess alcohol or recreational drug use, or hepatotoxic or over the counter medication usage were also enrolled following written, informed consent (REC #09/H0712/82). From December 2011 to January 2015 a further validation cohort of 101 patients (in two cohorts of 59 and 42 respectively) hospitalized with DC and 27 HC were recruited. The primary outcome was 90 day survival.

NMR data acquisition and processing

Plasma aliquots were thawed to room temperature. Aliquots of 200 µl plasma were added to 400 µl of a solution containing 0.9% NaCl and D_20 as previously described [34]. This was centrifuged at 12,000 g for 10 min and 550 µl of supernatant placed into 5 mm NMR tubes (Norell, Landisville, NJ, USA). Data were acquired in a random, blinded order on a Bruker Avance™ spectrometer (Bruker GmbH, Rheinstetten, Germany) with a 5 mm TXI probe operating at 600.13 MHz and 300 K. Data were acquired using a 1D technique using a standard pulse sequence with pre-saturation of the water resonance and Carr-Purcell-Meiboom-Gill (CPMG) spin echo sequences to attenuate the broader peaks arising from lipids and proteins, as described in Supplementary methods along with pre-processing techniques.

UPLC-TOF-MS data acquisition and processing

Plasma aliquots were thawed to room temperature. 50 µl of plasma were added to 150 µl of ice cold 100% methanol, vortexed briefly, and kept at −20 °C for 20 min. Following 10 min of centrifugation at 12,000 g, 170 µl of supernatant was transferred to Eppendorf™ containers (Eppendorf, Stevenage, UK). The supernatants were dried during 90 min of centrifugation under vacuum at 40 °C (Savant SpeedVac, ThermoScientific, Asheville, NC, USA). Dry material was suspended in 120 µl of high purity water (UpS grade, Romil Ltd, Cambridge, UK) and sonicated for 30 min. One-hundred µl of each sample was added in a random order in a 96-well plate while 20 µl were reserved from each to make a quality control (QC) sample. Samples were then transferred to the sample manager of a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) maintained at 4 °C. Reversed-phase chromatography was conducted using a gradient from acidified water to acidified methanol as detailed with MS conditions in Supplementary material. Extraction of features detected by mass spectrometry across the entire sample set was performed using XCMS software [35] operating in the R computing environment. A data matrix of samples analysed *vs.* detected features and corresponding intensity values was produced and analysed by multivariate analysis using SIMCA P software (v 12.0.1 Umetrics AB, Umeå, Sweden).

Multivariate analysis of the plasma spectral profiles

For both NMR and UPLC-TOF-MS data, principal components analysis (PCA) was performed to visualise any inherent clustering and identify outliers. Orthogonal projection to latent structure (OPLS) analysis was performed to maximise class differences while minimising variability unrelated to class. The R^2 value was calculated to give a measure of the goodness-of-fit or amount of variability