Published in final edited form as:

Liver Int. 2014 October ; 34(9): 1428–1444. doi:10.1111/liv.12541.

Integrated metabolomic profiling of hepatocellular carcinoma in hepatitis C cirrhosis through GC/MS and UPLC/MS-MS

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Abstract

Background & Aims—The metabolic pathway disturbances associated with hepatocellular carcinoma (HCC) remain unsatisfactorily characterized. Determination of the metabolic alterations associated with the presence of HCC can improve our understanding of the pathophysiology of this cancer and may provide opportunities for improved disease monitoring of patients at risk for HCC development. To characterize the global metabolic alterations associated with HCC arising from hepatitis C (HCV)-associated cirrhosis using an integrated non-targeted metabolomics methodology employing both gas chromatography/mass spectrometry (GC/MS) and ultrahigh-performance liquid chromatography/electrospray ionization tandem mass spectrometry (UPLC/MS-MS).

Methods—The global serum metabolomes of 30 HCC patients, 27 hepatitis C cirrhosis disease controls and 30 healthy volunteers were characterized using a metabolomics approach that combined two metabolomics platforms, GC/MS and UPLC/MS-MS. Random forest, multivariate statistics and receiver operator characteristic analysis were performed to identify the most significantly altered metabolites in HCC patients vs. HCV-cirrhosis controls and which therefore exhibited a close association with the presence of HCC.

Results—Elevated 12-hydroxyeicosatetraenoic acid (12-HETE), 15-HETE, sphingosine, γ -glutamyl oxidative stress-associated metabolites, xanthine, amino acids serine, glycine and aspartate, and a-cylcarnitines were strongly associated with the presence of HCC. Elevations in bile acids and dicarboxylic acids were highly correlated with cirrhosis.

Conclusions—Integrated metabolomic profiling through GC/MS and UPLC/MS-MS identified global metabolic disturbances in HCC and HCV-cirrhosis. Aberrant amino acid biosynthesis, cell turnover regulation, reactive oxygen species neutralization and eicosanoid pathways may be

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Conflict of interest: The authors do not have any disclosures to report.

hallmarks of HCC. Aberrant dicarboxylic acid metabolism, enhanced bile acid metabolism and elevations in fibrinogen cleavage peptides may be signatures of cirrhosis.

Keywords

Cirrhosis; HCC; liquid chromatography/mass spectrometry; metabolic profiling; Metabolomics

Hepatocellular carcinoma (HCC) is the world's third most lethal cancer, possessing a 5-year survival rate of 10% that results in between 2 50 000 to 1 000 000 deaths per year (1, 2). HCC culminates from a pre-existing long-term condition of cirrhosis in 90% of cases (3). Despite the risk factors being well characterized, the early detection of HCC that is crucial for curative intervention remains a significant challenge. This is in part because of the limited understanding of the mechanisms involved in the emergence of cancerous lesions within the cirrhotic microenvironment. Consequently, the majority of HCC patients are diagnosed after the cancer has already progressed to an advanced stage that possesses a grim outlook for curative intervention (4). Given that the risk factors for hepatocellular carcinoma are well known, studies comparing molecular phenotypes of patients with HCC and patients with cirrhosis can help improve the understanding of the pathophysiology of HCC and lay early groundwork for improved strategies in the monitoring of the at-risk population for HCC.

Recent advances in analytical chemistry have placed metabolomics at the frontier of disease phenotype characterization and lead marker generation. Metabolomics is the identification and quantification of all small molecules <1 kD in a tissue sample (5). Metabolomics couples liquid or gas chromatography/mass spectrometry (LC/MS, GC/MS) or nuclear magnetic resonance (NMR) with mass spectral and biostatistical analysis software to provide a validated and high-throughput method for obtaining and comparing the full complement of metabolites in tissue samples between groups. Because the liver is the principal organ of amino acid, lipid and carbohydrate metabolism and given the stepwise hepatocarcinogenic process involving a transition from cirrhosis to HCC, HCC is an ideal candidate for metabolomic profiling studies. HCC metabolomics has gained traction in recent years and at least 17 human HCC metabolomics studies have been reported (6-22). While these studies show possible roles for lysophospholipids (LPC), amino acids and glutathione metabolites in HCC development, significant heterogeneity in metabolomic data exists among these studies, likely attributable to differences in study design. Most of these studies identify metabolite expression profile differences between HCC patients and healthy volunteers, with only seven of these studies reporting the metabolomic profile differences between HCC patients and patients with cirrhosis (6, 7, 9, 14-17). Because greater than 90% of HCCs are diagnosed in patients with cirrhosis and given that HCC disease-monitoring paradigms target individuals with advanced fibrosis or cirrhosis, the metabolomic profile comparison of HCC vs. cirrhosis may be a more clinically relevant comparison than HCC patients vs. healthy subjects.

Only three previous metabolomics studies reported Model of End-Stage Liver Disease (MELD) and Child-Pugh liver function scores (14, 15, 18), leaving unrestrained the potential influence of decompensated liver function on metabolomic data. Body mass index

(BMI), a parameter directly related to metabolite expression levels, was also unaccounted for in all but one HCC metabolomics study (21), likely further confounding interpretation of metabolomic data. The previous metabolomics studies also employed a single technology of GC/MS or LC/MS or NMR. Because no single platform can provide global metabolomic coverage, an integrated metabolomics approach harnessing multiple platforms is needed to achieve a comprehensive identification of the metabolic underpinnings of hepatocarcinogenesis.

In this study, we characterized the metabolic disturbances associated with the presence of HCC using an integrated non-targeted metabolomics approach that employed both gas chromatography/mass spectrometry (GC/MS) and ultrahigh-performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC/MS-MS). The global serum metabolomes of a well-characterized and matched cohort of patients with HCC arising from hepatitis C (HCV)-associated cirrhosis, HCV-cirrhosis disease control patients (DC) and normal healthy controls (NHC) were determined through combined serum analysis with these platforms. The random forest supervised class prediction model, significance testing, false discovery rate, fold difference comparisons and receiver operator characteristic analysis were performed to identify metabolite expression trends most closely associated with HCC presence. We focused the metabolomic comparisons on HCC vs. DC to identify pathways most reflective of hepatocarcinogenesis in the environment of cirrhosis. In addition, through comparison of DC and NHC metabolomes, we identified metabolic pathways potentially contributing to the presence of cirrhosis. We also performed receiver operator characteristic (ROC) analysis to further gauge the ability of metabolites that were most significantly associated with HCC and cirrhosis to accurately place subjects into their appropriate groups. Our study reveals significant aberrations in a number of pathways potentially involved in the progression from hepatitis C-associated cirrhosis to HCC including reactive oxygen species homeostasis pathways, lipid signalling cascades, cell turnover regulation pathways and pathways of amino acid metabolism. We also identified possible signatures of HCV-cirrhosis that included pathways of bile acid, dicarboxylic acid and fibrinogen cleavage metabolism.

Materials and methods

Patients

This study was approved by the University of Florida Institutional Review Board and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983. This study cohort included 30 baseline HCC patients whose cancer arose exclusively from HCV-cirrhosis, 27 HCV-cirrhosis disease control patients (DC) and 30 healthy non-diabetic controls (NHC). We recruited only HCC and DC patients with histologically proven HCV-related cirrhosis given that HCV-cirrhosis is the leading cause of HCC in Western countries. Simultaneous HCV and hepatitis B infection was absent in all study participants. HCC patients were diagnosed and staged according to the Barcelona Clinic Liver Cancer (BCLC) staging system. Patient clinical characteristics were obtained from the medical record and included the following parameters: Demographical information, body mass index (BMI), alpha-fetoprotein (AFP) level, Child–Pugh score (CP) and Model for End-Stage Liver

Disease score (MELD). Serum samples were collected from patients with HCC at baseline and all were naïve to cancer treatment at the time of blood withdrawal. All DC patients were enrolled in our surveillance programme, received serial cross-sectional imaging every 6 months and had no liver masses on enrolment. Because the metabolomic differences between HCC patients and HCV-cirrhosis controls are not well established, the absence of regenerative nodules in the DCs allows for the identification of conspicuous metabolic differences between these groups. NHCs were recruited from the LifeSouth Community Blood Center in Gainesville, FL. Blood was drawn from patients presenting to clinic after overnight fast, whole blood samples were immediately processed for serum and serum samples were stored at -80°C until retrieval.

Metabolomics instrumentation

For metabolomic profiling of patient serum, we employed an integrated, non-targeted GC/MS and UPLC/MS-MS metabolomics system described in a previous publication (23). The metabolomics platform utilized three operation modes to ensure full coverage of the metabolome: Gas chromatography–mass spectrometry (GC-MS) to detect lipids and organic phase biomolecules, UPLC/MS-MS in the positive electrospray ionization (ESI) mode optimized for detection of basic species and UPLC/MS-MS in the negative ESI mode optimized for detection of acidic species. In the GC/MS arm of this study, samples were separated and quantified on a Thermo–Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer (ThermoFisher Corporation, Waltham, MA, USA) using electron impact ionization. The UPLC/MS-MS portion of the platform was based on a Waters Acquity UPLC (Waters, Millford, MA, USA) and a Thermo–Finnigan linear trap quadrupole fourier transform (LTQ-FT) mass spectrometer using electrospray ionization with a linear ion trap front end and a Fourier transform ion cyclotron resonance mass spectrometer backend.

Sample preparation and run specifications

The sample preparation process was carried out using a Hamilton MicroLab STAR automated liquid handler (Hamilton Robotics, Reno, NV, USA). 200 μl aliquots of serum from each study subject, stored at -80°C , were thawed on ice and each aliquot was split into three equal parts: One aliquot for GC/MS, one aliquot for the ESI-positive mode of the UPLC/MS-MS analysis and the third aliquot for the ESI-negative mode. Recovery standards were added prior to the first step in the extraction process for monitoring of extraction efficiency and authenticated retention index (RI) markers were also spiked into each sample and allowed for analyte RI determination. Samples were then evacuated into a collection chamber in vacuo. Sample preparation was conducted using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. Samples were placed briefly on a TurboVap® (Zymark, East Lyme, CT, USA) to remove the organic solvent. Each sample was then frozen and dried under vacuum. The resulting extract was divided into two fractions: one for analysis by LC and one for analysis by GC. Samples destined for GC/MS analysis were redried under vacuum desiccation for a minimum of 24 h prior to being derivatized under dried nitrogen using bistrimethyl-silyl-trifluoroacetamide (BSTFA). The GC column was 5% phenyl and a 40°C – 300°C temperature ramp was applied in a 16-min period. The sample extract destined for the LC-MS arm of the study was then itself equally split into two aliquots, one for the ESI-

positive mode and one for the ESI-negative mode, dried, then reconstituted in acidic or basic LC-compatible solvents. One aliquot was analysed using acidic conditions optimized for the detection of positive ions and the other aliquot was analysed in basic conditions optimized for the detection of negative species, and these extracts were monitored using separate acid/base dedicated 2.1 mm × 100 mm Waters BEH C18 1.7 μ m particle columns heated to 40°C. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol both containing 0.1% formic acid, while the basic extracts, which also used water/methanol, contained 6.5 mM ammonium bicarbonate. The MS interface capillary was maintained at 350°C with a sheath gas flow of five for the positive and the negative injections. For the positive injection, the spray voltage was 4.5 kV and for the negative injection the spray voltage was 3.75 kV. The MS scanned 99–1000 m/z with a mass resolving power set to 50 000, alternated between MS and MS/MS through dynamic exclusion to minimize redundant MS/MS scans, and was set for six scans per second. For MS, the ion trap fill time cut-off was 200 ms and for MS/MS, the ion trap fill time cut-off was 100 ms.

There were 13 retention markers employed in the LC-positive electrospray mode and 11 in the LC-negative mode, eluting every 30s of chromatography, and these markers were given a fixed RI that provided a linear reference for extrapolation of the analytes' elution times. This measure was done to control for potential interday and intraday variability in the process.

Data processing and analysis

The informatics system consisted of four major components, the Laboratory Information Management System (LIMS), the data extraction and peak-identification software, data processing tools for QC and compound identification, and a collection of information interpretation and visualization tools for use by data analysts. The hardware and software foundations for these informatics components were the LAN backbone and a database server running Oracle 10.2.0.1 Enterprise Edition. The LIMS system enabled fully auditable and secure automation of the metabolomics analytical process. The scope of the LIMS system encompassed sample accessioning, sample preparation and instrumental analysis and reporting, and advanced data analysis. Metabolite identification and quantification was performed in an automated fashion through mView software that was grounded in the LIMS data structure. Chromatographical peaks, retention time, mass/charge (m/z) data and MS spectra were obtained for all compounds in the serum samples. Mass spectra generated during the data curation process were automatically cross-compared by mView with spectra of over 2500 authenticated metabolite reference standards housed in an internal library. Extracted ion chromatograms were binned by mass in a given range, baseline noise was determined, and peak height, signal-to-noise, width, symmetry and area cut-offs were applied to the detected MS peaks. MS peaks that passed these threshold criteria were binned into a relational database for further analysis. Putative identification was automatically determined on the basis of peak characteristics (peak integration of analyte and reference standard), mass-to-charge ratio and retention index.

Positive identifications were based on analyte retention index of the analyte that was within 75 RI units of the reference standard (approximately 5 s); a mass match within 0.4 m/z , and

MS/MS forward and reverse scores of >80%. The MS/MS forward score describes the percentage of ions in the experimental MS/MS spectrum positively identified in the reference spectrum, and the reverse score describes the extent to which the fragmentation pattern of the reference spectra was present in the experimental MS/MS spectrum. Analytes not meeting these threshold parameters and which exhibited a forward and/or reverse score <35% were automatically rejected. The combination of chromatographical properties and mass spectra gave an indication of a match to the specific compound or an isobaric entity. Additional entities could be identified by virtue of their recurrent nature (both chromatographical and mass spectral) through statistical analysis. As an added quality assurance measure, spectral matches through software were confirmed by a trained mass spectrometry specialist. For ions with counts greater than 2 million, an accurate mass measurement was performed. Accurate mass measurements were made on the parent ion as well as fragments through co-introduction of endogenous authenticated reference standards. The typical mass error was less than 5 ppm.

Quality control

The GC/MS and UPLC/MS-MS samples were randomly sorted within each group and processed on three consecutive days such that each run consisted of exactly one-third of each group's samples. The mass spectrometer was tuned and calibrated for mass resolution and mass accuracy on a daily basis using authenticated reference standards. Process coefficients of variation involving instrument performance, chromatography and mass calibration were checked to ensure quality on a daily basis. The median relative standard deviation (RSD) of instrument variability was 5% and total process variability RSD was 12%. The QC and curation processes were designed to ensure accurate and consistent identification of true chemical entities and to remove those representing system artefacts, mis-assignments and background noise.

An extensively characterized pooled human plasma sample and a pooled matrix of all study samples were processed alongside the experimental samples to respectively assess whether the process was operating within established specifications and to distinguish biological variability from process variability. Also included in the analyses were ultrapure water blanks and solvent blanks, and these were used to assess the process contribution to signals and to identify potential contamination sources. Still other QC standards employed during the analysis included derivatization, internal and recovery standards. These were used to monitor variability of derivatization in the GC/MS arm of this study, assess variability and performance of instrument, and assess extraction efficiency respectively. A data normalization step was performed to correct variation resulting from instrument interday tuning differences. Essentially, each compound was corrected in run-day blocks by registering the medians to equal one (1.00) and normalizing each data point proportionately. Metabolites with areas that trended systematically upward or downward were automatically removed from the analysis.

Statistical analysis

Statistical analysis of metabolomic data was performed with the program 'R' (<http://cran.r-project.org>) and MedCalc (Mariakerke, Belgium). RF, a supervised machine-learning

classification model reporting on the consensus of a large number of decision trees, was employed to (i) predict HCC, DC and NHC classes on the basis of patients' global metabolomic expression profiles and (ii) identify metabolites most important to the group classification and hence exhibiting a strong correlation to the presence of HCC. Metabolites with the largest mean decrease accuracy – the magnitude loss of class prediction accuracy following removal of a metabolite from the random forest analysis – possessed the highest importance to the class prediction and were therefore deemed important to the presence of HCC. Following log transformation and variance normalization of the metabolite levels, intergroup fold differences for HCC vs. DC and DC vs. NHC were generated and a two-sided *t*-test was used to identify metabolites whose expression differed significantly ($P < 0.05$) between compared groups. The false discovery rate (*q*-value), or confidence in the *P*-value, for each metabolite was also determined. Unsupervised hierarchical clustering analysis of HCC and DC metabolomes was performed using dChip (Harvard, Cambridge, MA, USA). To validate the importance of metabolites for random forest, and to further gauge these metabolites' class prediction utility and potential association to HCC, we tested their potential predictive utility for HCC using ROC curve analysis. We also tested the correlation of pathways strongly disrupted in DCs vs. NHCs through ROC. The ROC analysis was performed using MedCalc using MS peak areas corresponding to metabolite concentration in each study subject. All metabolites analysed through ROC exhibited intergroup fold difference of >1.5 in the HCC vs. DC comparison and >10 in the DC vs. NHC comparison, and a significance level $P < 0.05$ and false discovery rate $q < 0.10$ for these fold differences.

Results

Patients

Most of our patients were Caucasian males and the mean ages of HCC patients and cirrhosis controls were 60 and 55 years respectively (Table 1). Our HCC group included patients with stage A ($n = 13$), B ($n = 10$) and C ($n = 7$). Twenty eight of thirty HCC patients had a BMI <30 , the USA obesity cut-off, a study inclusion criteria that minimized the influence of excess adiposity on the generated biochemical profiles. The majority of HCC and DC patients had an AFP level <400 ng/ml.

Metabolomic profiles

The metabolomics analysis detected a total of 485 biomolecules. One hundred and seven metabolites were significantly altered in HCC vs. DC ($P < 0.05$), but none of these metabolites exhibited >3 -fold elevation or downregulation in this comparison. In contrast, more patent fold differences were witnessed in the DC vs. NHC comparison, with 245 metabolites exhibiting significantly altered levels between these groups and eight among them exhibiting >10 -fold overexpression in the cirrhosis patients. The most differentially expressed metabolites in HCC patients vs. DCs belonged to pathways of amino acid metabolism, eicosanoid signalling, lipid metabolism, acylcarnitine metabolism, nucleotide metabolism and oxidative stress homeostasis (Table 2a). Pathways of importance for cirrhosis were bile acid metabolism, acylcarnitine metabolism, dicarboxylic fatty acid metabolism, fibrinogen peptide cleavage cascades, haemoglobin catabolism and dipeptide

metabolism (Table 2b). Box plots for selected metabolites provide an additional depiction of intergroup metabolite expression levels (Figure S1).

Pathways involved in stepwise hepatocarcinogenesis

Lipid metabolism—The liver can be considered the hub of lipid metabolism and not surprisingly, numerous HCC metabolomics and genomics analyses have implicated aberrations in lipid metabolism as signatures of HCC development (7, 10, 18, 20, 24). In our study, we witnessed a strongly significant upregulation of multiple lipid classes in HCC vs. DC and NHC and in DC vs. NHC (Table 2a). Monohydroxy fatty acids, dicarboxylic fatty acids (DCA), glycerophospholipids, sphingolipids and acylcarnitines all trended higher in HCC vs. DC. This is in contrast to what would be expected from BMI data for the respective groups showing greater adiposity in DC vs. HCC. An especially remarkable upregulation of DCAs was seen in the comparison of cirrhosis controls vs. NHC (Table 2b), where non-anedioate (azelate), undecanedioate and decanedioate (sebacate) showed a strongly significant respective overexpression of 54-, 36- and 26-fold in cirrhosis vs. NHC. We also witnessed global downregulation of two major glycerophospholipid classes, the glycerophosphoethanolamines and the glycerophosphocholines, in DCs vs. NHC (Figure S1). Medium chain fatty acid levels were not statistically different in HCC vs. DC, but this class of lipid metabolites was strongly upregulated in DC vs. NHC. A massive downregulation of LPCs was also witnessed in DC vs. NHC and HCC vs. NHC, a trend observed in numerous HCC metabolomics studies (10, 19, 20, 22). Of the 33 LPCs detected, 28 were significantly downregulated in DC vs. NHC and 23 were significantly diminished in HCC vs. NHC. There was an increase of LPCs in HCC vs. DC, but this was not significant ($P = 0.21$).

Bile acids—Bile acids, which are routinely implicated as markers of liver disease in HCC metabolomics studies (17-20), exhibited vast overexpression in cirrhosis patients vs. NHCs. Taurochenodeoxycholate (TCDCA), taurocholate (TCA), glycohyocholate (GHCA) and glycocholate (GCA) were 17.8-, 15-, 6.6- and 4-fold upregulated in DC vs. NHC respectively.

Acylcarnitines—Alone, fatty acyl-CoAs (fatty acids) cannot enter the mitochondria for β -oxidation and must first be converted to acylcarnitines by enzymes of the carbamoyl phosphate transport shuttle system. In our study, acylcarnitine, glutaryl carnitine and succinyl carnitine were significantly upregulated in HCC patients vs. DCs. In DC vs. NHC, medium chain acylcarnitines averaged two-fold overexpression, while long chain acylcarnitines trended downward in HCC vs. DC.

Lipid signalling molecules—Eicosanoids are 20-carbon autocrine and paracrine lipid signalling molecules that perform a myriad of signal transduction roles, notably in the cyclooxygenase (COX) and lipoxygenase (LOX) pathways, and these metabolites are best known for regulating pain and inflammation. Eicosanoids consist of leukotrienes, prostaglandins and thromboxanes and are derived from arachidonic acid or linoleate. In our study, eicosanoids were significantly overexpressed in HCC patients vs. DCs and downregulated in DCs vs. NHC (Table 2a). These differences included the two

prostaglandin parent molecules arachidonate (20:4n6), which was significantly elevated in HCC vs. DC, but downregulated in DC vs. NHC, and dihomolinoleate (20:2n6), which also showed significant elevation in HCC vs. DC. Linoleate/arachidonate-derived lipid signalling molecules 13-HODE+9-HODE (2.54-fold increase in HCC:DC), 15-HETE (2.8 ↑) and 12-HETE (1.7 ↑) exhibited elevation in HCC vs. DC.

Oxidative stress homoeostasis—The liver is the major site of glutathione (GSH) synthesis and is the principal organ of oxidative stress homoeostasis. Key HCC vs. DC trends among oxidative stress-associated metabolites included 1.62-fold upregulation of xanthine ($P = 9.35 \times 10^{-5}$), a purine degradation intermediate whose production is accompanied by generation of reactive oxygen species (ROS) hydrogen peroxide (H_2O_2) and 1.56-fold elevated 2-hydroxybutyrate (2HB) ($P < 0.0003$), an important neutralizing agent of reactive oxygen species that complements glutathione during periods of overwhelming oxidative stress. γ -glutamyl peptides, which are liberated via γ -glutamyl trans-peptidase-mediated breakdown of glutathione, were also significantly upregulated in HCC vs. DC.

Unknown metabolites associated with clotting cascade—As the site of prothrombin production, the liver plays an important role in the clotting cascade, and blood clotting is sometimes measured in concert with liver function tests. Prothrombin is processed to thrombin, a serine protease that cleaves fibrinogen to fibrin, leading to clotting. Our study identified two unknown fibrinogen cleavage fragments, denoted by amino acid sequence, that were strongly upregulated in DC vs. NHC. ADSGEGDFXAEGGGVR (ADS) differed from DSGEGDFXAEGGGVR (DSG) by the presence of an additional alanine on the N-terminus. DSG and ADS exhibited 11.8- and 2.9-fold elevation in DC vs. NHC respectively. DSG levels were further reduced 35% in HCC vs. DC ($P = 0.046$), possibly suggesting altered fibrinogen peptide metabolism with HCC.

Amino acids and protein metabolites—Numerous studies have linked amino acid metabolism aberrations to cancer development (7-11) including several recent HCC metabolomics studies (20-22). Consistent with these reports, we found significant upregulation of serine, aspartate, glycine, phenylalanine and glutamate in HCC vs. HCV-cirrhosis, and these metabolites had the strongest P -values and lowest false discovery rates in the intergroup metabolite expression level comparison between HCC patients and DCs. Serine was the most significantly altered amino acid between HCC patients and DCs ($P = 7.0 \times 10^{-6}$). A more reactive form of serine, homoserine, also trended higher with 1.62-fold overexpression ($P = 0.0002$) in HCC vs. DC. Glycine was also strongly associated with HCC presence, exhibiting a 1.36-fold increase in HCC patients vs. DCs ($P < 2.58 \times 10^{-5}$). The trend of overexpressed amino acids in HCC vs. DC was further seen among amino acids valine ($P = 0.0409$), tyrosine ($P = 0.0211$), asparagine ($P = 0.0108$) and tryptophan ($P = 0.0036$).

The reverse trend in amino acid expression was true for the comparison between DCs and NHCs, with HCV-cirrhosis patients exhibiting strongly significant downregulation of serine, glycine, aspartate, glutamate and phenylalanine. We also observed a consistent downregulation of dipeptides pyroglutamylglycine, glycylvaline, alanylalanine and

aspartyleucine in DC vs. NHC (Table 2a). In HCC vs. DC, pyroglutamylglycine and glycylvaline trended 1.95 and 1.48 times higher, and of the only four metabolites that were significantly downregulated in HCC vs. DC, three were dipeptides: Phenylalanyltryptophan, phenylalanylserine and leucyl-alanine.

Other notable metabolic alterations—Haemoglobin catabolites bilirubin and biliverdin are routinely implicated as important class distinguishers in HCC metabolomics studies (10, 13, 20, 22), and elevated bilirubin is a clinically useful hallmark of liver decompensation (25). Consistent with its implicated role in advanced liver disease, we observed significantly elevated levels of haeme catabolites bilirubin and elevations in biliverdin and urobilinogen in DC vs. NHC and HCC vs. NHC. There was not, however, a significant difference between the levels of haeme catabolites in HCC patients vs. DC. 2-pyrrolidinone, a γ -aminobutyric acid (GABA) metabolite with an unknown role in cirrhosis or cancer, exhibited a strongly significant, progressive elevation from NHC to cirrhosis and cirrhosis to HCC. 1,2-propanediol, a ketogenesis intermediate, was 5.8 times upregulated in DC vs. NHC ($P < 1.0 \times 10^{-15}$). We also witnessed significant elevation of purine and pyrimidine metabolites in HCC vs. DCs. And, whereas the N-acetyl amino acids exhibited a consistent downregulation in cirrhosis patients vs. healthy volunteers, we observed global upregulation of N-acetyl amino acids in HCC vs. DC (Table 2a).

Random forest class prediction model

Random forest, a supervised class prediction model, was performed to (i) determine the capacity for global metabolomes to accurately classify patients into their respective groups and (ii) to identify metabolites most important to the class prediction and hence which possessed the strongest correlation to the respective disease. In the random forest supervised class prediction analysis comparing all three groups, RF accurately classified 100% of the NCs, 70% of DCs and 70% of HCC patients (Fig. 1a), corresponding to a mean prediction accuracy of 80%. For the RF comparison of HCC vs. cirrhosis (Fig. 1b), RF accurately placed 22/30 HCC patients into their appropriate group and 19/27 cirrhosis controls into the DC group. This predictive performance of 72% is a satisfactory improvement over the 50% accuracy that would be expected from random assignment to either group. We also performed hierarchical cluster analysis to construct an unsupervised class prediction model of our metabolomic data (Figure S2), but this analysis identified no notable trends.

Receiver operator characteristic curve analysis

The collective results of random forest, significance testing, fold-difference comparisons and false discovery rate analysis identified several metabolites that were strongly associated with HCC and cirrhosis. To validate these metabolites' importance to the random forest analysis and to further characterize the capacity for these metabolites to accurately stratify subjects into their appropriate groups, we performed ROC analysis using MS peak areas. The HCC vs. DC comparison was used to construct ROC curves for metabolites exhibiting a significant correlation to HC, and the DC vs. NHC comparison was used for characterizing the predictive value of these metabolites for cirrhosis (Table 3). Aspartate was the most sensitive metabolite for distinguishing HCC patients from cirrhosis controls, possessing a sensitivity of 100 and a specificity of 52.0 for HCC (area under the curve, AUC = 0.789, $P <$

0.0001). In comparison, AFP, the principal diagnostic biomarker for HCC used in clinical disease monitoring, had a sensitivity of 63.3 and a specificity of 83.6 for detecting the presence of HCC in the same patient cohort (AUC = 0.760, $P < 0.0001$) (Table 3a). Serine had a sensitivity of 73.3 and a specificity of 85.2 when used to distinguish HCC patients from DCs (AUC = 0.831, $P < 0.0001$). Serine's sensitivity for HCC was enhanced when used to distinguish early HCC patients (stage A, $n = 13$) from the disease controls, possessing a sensitivity and specificity of 76.9 and 88.9 respectively (AUC: 0.875, $P < 0.0001$). Glycine also had a high sensitivity of 83.3 and a specificity of 63.0 (AUC = 0.801, $P < 0.0001$) for HCC.

Xanthine, a purine degradation metabolite accompanied by production of ROS hydrogen peroxide (H_2O_2), had a sensitivity of 63.3 and a specificity of 88.9 for detecting HCC presence (AUC = 0.790, $P < 0.0001$). The 15-lipoxygenase product 15-HETE, a lipid signalling eicosanoid involved in inflammation and endothelial cell adhesion, was also identified as a potentially important metabolic signature of HCC, possessing a sensitivity of 83.3 and a specificity of 59.3 for HCC presence (AUC = 0.705, $P = 0.0011$). Consistent with aforementioned reports implicating alterations in lipid metabolism in HCC (7), our study revealed numerous lipid metabolites that were significantly overexpressed in HCC vs. DC. Of the lipid metabolites, 2-hydroxypalmitate (2HP), with a sensitivity of 80 and a specificity of 63, was the most accurate distinguishing lipid marker between HCC patients and cirrhosis controls (AUC = 0.736, $P < 0.0004$). Given the previously implicated role of cell turnover regulator sphingosine 1-phosphate in HCC progression, we tested its utility as a predictor of the presence of HCC and found that it possessed a sensitivity of 58.3 and a specificity of 86.7 for distinguishing HCC patients from DCs (AUC = 0.731, $P < 0.0058$).

Receiver operator characteristic analysis also identified several metabolites exhibiting potential as signature markers of cirrhosis (Table 3b). Given the striking upregulation of dicarboxylates and bile acids in DC vs. NHC and similar patent expression differences among other metabolites in DC vs. NHC (Table 2b), we performed ROC analysis on metabolites most closely associated with the presence of cirrhosis to determine the sensitivity and specificity of these metabolites in accurately classifying patients in the study cohort into their appropriate groups. The results of this ROC analysis (Table 3b) reveal that azelate, which was 54 times upregulated in cirrhosis patients vs. NHC, possessed 100% sensitivity, 100% specificity and AUC = 1.00 ($P = 0.00$) when used to distinguish DC from NHC. Sebacate was elevated 26-fold in DC vs. NHC and ROC analysis showed that this DCA also possessed a sensitivity of 100% and a specificity of 100% with an AUC = 1.00 and $P = 0.00$ when used to discriminate between these groups. A third DCA, undecanedioate, was elevated 36-fold in DC vs. NHC and possessed a sensitivity of 78% and a specificity of 90% (AUC = 0.860, $P < 0.0001$) when employed in the stratification of DCs from NHCs. Two other DCAs, 2-hydroxyglutarate and hexadecanedioate, likewise exhibited strong sensitivity and specificity in accurately distinguishing cirrhosis patients from healthy volunteers. Finally, GABA metabolite 2-pyrrolidinone, which showed a significant positive trend from NHC through DC and to HCC, possessed 100% sensitivity and 100% specificity (AUC = 1.00, $P = 0.00$) for distinguishing DCs from NHCs.

Discussion

In this study, integrated GC/MS and UPLC/MS-MS metabolomic profiling identified significant aberrations in eicosanoid, lipid, amino acid, ROS and nucleotide metabolism in patients with HCV-cirrhosis-related HCC compared to patients with HCV-cirrhosis. Arachidonic acid-derived eicosanoids 12-HETE and 15-HETE, several γ -glutamyl peptides, cell turnover metabolite sphingosine, amino acids serine and glycine, and several acylcarnitines were most significantly associated with HCC.

Potential role of significantly altered pathways in HCC

12-HETE and 15-HETE are products of lipoxygenase and cytochrome P450 enzymes and exert both pro and anti-inflammatory effects (26). 12-HETE is reported to augment tumour metastatic potential through activation of protein kinase C (27). 12-LOX has been shown to be elevated in HCC tumours and in HepG2 and L02 HCC cell lines (28), and studies show attenuation of HCC proliferation by 12-LOX inhibitor baecalein (29). The Endogenous production of 12-HETE has also been observed in solid tumours (stomach, lung colon, melanoma) and has been shown to enhance the potential for tumour cell metastasis (30). 15-HETE was recently shown to promote HCC growth and metastasis through the phosphoinositide 3-kinase/protein kinase B/heat shock protein 90 (PI3K/Akt/HSP90) pathway (31). In our study, 12-HETE and 15-HETE were significantly downregulated in DC vs. NHC, but experienced a rebound in expression in the HCC group, with both compounds exhibiting significant elevation in HCC vs. DC. Their re-emergence in HCC suggests potential roles for LOX or cytochrome P450 pathways in HCC development.

Oxidative stress through accumulation of ROS species superoxide, hydrogen peroxide (H_2O_2) and hydroxyl radical is known to promote the development of HCC primarily by enhancing DNA damage (32). Consistent with a previous HCC metabolomics study implicating enhanced γ -glutamyl peptide expression in HCC (13), our data reveal significant elevation of oxidative stress-related metabolites xanthine and several γ -glutamyl peptides in HCC vs. DC. Xanthine is produced from hypoxanthine by xanthine oxidase, and the production of xanthine is accompanied by generation of H_2O_2 . One study reported a significant elevation of xanthine oxidase activity in HCC (33), while a second study found a decrease in XO activity in HCC (34). The concomitant elevation of hypoxanthine in our HCC vs. DC comparison suggests overactivity of xanthine oxidase in HCC. We hypothesize that the enhanced production of xanthine resulting in elevated H_2O_2 results in greater oxidative stress that promotes the development of HCC. Interestingly, one study showed that the elevation of ROS in hepatoma cells was a prerequisite for heightened glycolysis (35), consistent with the Warburg theory of cancer. This elevation of ROS in HCC vs. cirrhosis may reflect increased oxidative stress that is a hallmark of the neoplastic microenvironment in HCC and may also synergistically promote the enhanced glycolysis exhibited by growing tumours.

The finding of elevated γ -glutamyl peptides in HCC vs. DC further reinforces the theory of increased oxidative stress promoting the development of HCC. γ -glutamyl peptides are the building blocks of glutathione, the body's chief antioxidant that is produced primarily in liver and acts as a reducing agent to neutralize ROS. Glutathione is a byproduct of γ -

glutamylpeptidase (GGT), an enzyme serving as a clinical signature of chronic liver disease. GGT breakdown of glutathione results in the liberation of free γ -glutamyl peptides. Glutathione scavenging of ROS is catalysed by glutathione S-transferase P1 (GSTP1) and studies show that impairment of GSTP1 promotes HCC (36, 37). Another oxidative stress metabolite, 2-hydroxybutyrate, was also strongly elevated in HCC vs. cirrhosis. When overwhelming oxidative stress eclipses glutathione antioxidant capacity, the liver produces antioxidant 2HB through an 'emergency' transsulfuration pathway (38). The upregulation of 2HB may be a signature of elevations in ROS that summon emergency LDH production of 2HB to complement the antioxidative effects of GSH. ROC analysis of Xanthine, 2HB and the γ -glutamyl peptides demonstrates that these oxidative stress metabolites may show promise as ROS-related metabolic markers of HCC.

Our RF and ROC analyses showed that in the comparison of HCC and DC metabolomes, serine, aspartate and glycine were most strongly associated with the presence of HCC. Further, we observed a strongly significant downregulation of serine, glycine, aspartate, glutamate and phenylalanine in cirrhosis patients vs. healthy controls, consistent with the notion that an emergent HCC would intensify anabolic demands. Our finding of elevated amino acids in HCC coincides with the findings of other HCC metabolomics works (6, 18). The elevation of amino acids in HCC may also suggest possible cancer-relevant regulatory roles. Serine's importance to HCC development may be linked to its secondary function as an allosteric activator of pyruvate kinase M2 (PKM2). PKM2 is the principal cancer isoform of PK involved in the rate-limiting conversion of phosphoenolpyruvate to pyruvate during glycolysis. Serine not only activates this enzyme upon binding, but depletion of serine results in an attenuation of PKM2 activity (39). Glycine is also a vital anabolic metabolite of growing cancers and was recently shown to be elevated in an HCC metabolomics study (12). A large-scale metabolic profile analysis of 60 cancer cell lines using LC/MS-MS found that glycine biosynthesis was among the most important pathways for the development of a broad spectrum of cancers (40). Jain *et al.* further demonstrated that radioactively labelled glycine was incorporated in the purine nucleotides of the rapidly proliferating cancer cells. Consistent with this observation, we observed significant upregulation of several purine and pyrimidine metabolites in HCC vs. DC, including a near two-fold elevation of adenine and less marked but still significant upregulation of uridine and xanthine in HCC vs. DC. The elevation of adenine in HCC is in agreement with the increased metabolic needs of emerging lesions. The downregulation of uridine and xanthine in DC vs. NHC followed by increased levels of these nucleotides in HCC supports the principle of metabolic enhancement during HCC development.

The deregulation of lipid metabolism seen in HCC vs. DC is consistent with multiple HCC metabolomics studies (7, 16, 17). Notably, the significant elevation of sphingosine in HCC patients vs. DCs ($P = 0.0017$) in our study was previously reported in a HCC metabolomics study (17). Sphingosine is produced from ceramide in a reversible manner by acid ceramidase (AC) (41). The phosphorylated form of sphingosine, sphingosine 1-phosphate, is routinely implicated as an inducer of cancer growth. The ceramide/sphingosine/sphingosine-1-phosphate reaction is known as the 'sphingosine rheostat' (42), a pivotal cell turnover homeostasis regulatory pathway. The observed elevation in sphingosine in HCC

patients vs. cirrhosis controls may reflect the establishment of bolstered cell survival capacity in HCC mediated through enhanced acid ceramidase pathway activity. Interestingly, the initiation of the sphingosine production pathway involves the combination of palmitoyl-CoA and serine via serine palmitoyltransferase to produce the biosynthetic intermediate 3-ketosphinganine. The observed elevation in serine levels in our HCC patient group may reflect not only the tumour's heightened biosynthetic needs and serine's enhancement of HCC proliferation through PKM2, but also the heightened activity of pathways such as AC. The inhibition of AC resulting in accumulation of ceramide/truncation of sphingosine levels has been shown to both sensitize HCC cells to chemotherapy and shrink HCC tumours *in vivo* (43). Gene expression profiling of hepatitis C-induced HCC by Mas and colleagues showed that AC was among the top 10% of the most overexpressed genes in HCC samples vs. normal uninvolved liver tissues (44). Other studies aimed at sphingosine kinase inhibition have shown abrogation of HCC development when used in combination with sorafenib (45, 46). Our metabolomics data are in agreement with a growing body of evidence suggesting that therapies targeting the ceramide/sphingosine axis may be beneficial in abrogating HCC tumour growth.

Potential role of significantly altered pathways in cirrhosis

Receiver Operator Characteristic analysis of dicarboxylic acids and bile acids, metabolites that were significantly overexpressed in DCs vs. NHCs, showed that these markers were highly sensitive and specific for cirrhosis in this study cohort. The liver biopsy is a standard but invasive approach to diagnose cirrhosis (47). While novel strategies are emerging to detect the presence of subclinical cirrhosis, new non-invasive biomarkers that accurately identify advanced fibrosis or early cirrhosis are needed. We observed a significant overexpression of dicarboxylic acids in DC vs. NHC and ROC data of azelate and sebacate show that each of these metabolites accurately distinguished DCs from NHCs with 100% sensitivity and 100% specificity (AUC = 1.00, $P = 0.00$). DCAs are toxic very long chain fatty acids that inhibit mitochondrial β -oxidation and serve as substrates for PPAR- α β -oxidation (48). The overexpression of DCAs in DCs vs. NHC and HCC vs. NHC may be related to the impairment of the CPT shuttle system as shown by the build-up of acylcarnitines in DC vs. NHC. In lieu of a deregulation in CPT function, PPAR- α activity would be enhanced to compensate for the loss of mitochondrial β -oxidation, and this enhancement is shown to promote HCC development (49-51). Given that DCAs are substrates of PPAR- α β -oxidation, their elevation may be a signature of a metabolic shift from mitochondrial to peroxisomal fatty acid oxidation. The injurious effects of a shift from mitochondrial to PPAR- α β -oxidation involve the fact that H_2O_2 , a ROS, is liberated with the oxidation of every DCA during PPAR- α β -oxidation. Overreliance on this pathway may increase the oxidative stress, contributing to greater likelihood of cell death from accumulating levels of free radicals.

Further aberrations in lipid metabolism were witnessed through strong elevations in bile acids TCDCA, TCA, GHCA and GCA in DC vs. NHC. ROC analysis confirmed that these bile acids possessed strong diagnostic utility for cirrhosis. Several studies have shown that total bile acids have high diagnostic utility for cirrhosis and hepatobiliary disease (52-54). Other significantly elevated markers of cirrhosis included two unknown fibrinogen cleavage

byproducts. The accelerated breakdown of fibrinogen results in reduced clotting capacity in liver disease patients and is strongly implicated in cirrhosis (55). The elevation of fibrinogen catabolites in DC vs. NHC is in agreement with the increased prothrombin time (INR) that is a signature of impaired extrinsic pathway of coagulation in patients with advanced liver disease. Highly overexpressed acylcarnitines in both HCC vs. DC and DC vs. NHC were also observed and these trends are consistent with recent HCC metabolomics studies showing an elevation of these metabolites in chronic liver disease patients. Aberrant acylcarnitine metabolism has been implicated as an important mechanism of cirrhosis onset (56, 57). Increases in acylcarnitines in HCC patients vs. NHC and cirrhotics vs. NHCs may reflect a failure in carbamoyl phosphate transferase I (CPTI) catalysed unification of free fatty acid with carnitine enabling mitochondrial β -oxidation and hence may explain the strong lipid signature in DC vs. NHC and HCC vs. DC. We also observed consistent downregulation of two major glycerophospholipid classes in DC vs. NHC, the glycerophosphoethanolamines and the glycerophosphocholines, and a continuation of this significant downward trend through cirrhosis into HCC (Figure S1). The sudden decrease of these metabolites in HCV-cirrhosis, followed by further decreases in HCC vs. DC, suggests impairment of the mechanisms that yield these metabolites or a metabolic shift that favours hyperconsumption of these metabolites during advanced liver disease. The role of these lipid metabolites in hepatitis C, cirrhosis and HCC is not well known.

Conclusion

Through an integrated metabolomic profiling approach utilizing GC/MS and UPLC/MS-MS, we identified metabolic pathway disturbances that may be associated with the presence of HCV-cirrhosis-related HCC. In the comparison of DC vs. NHC, we also identified metabolites exhibiting promise as signatures of the presence of HCV-cirrhosis. Nearly all HCC metabolomics studies employ a single metabolomics platform, but a limitation in this strategy is that no single metabolomics platform can achieve global coverage of all metabolites in a tissue sample. The novelty in our approach is that through integration of GC/MS and both electrospray modes of UPLC/MS-MS, we identify metabolites belonging to all metabolic classes (lipids, amino acids, carbohydrates and nucleotides), accomplish global metabolite coverage, and provide a more comprehensive glimpse at the metabolic underpinnings of HCC. We also matched patients by demographical and clinical characteristics relevant to liver performance status (MELD, Child-Pugh) to control for factors that may confound interpretation of the metabolomic data. In the referenced HCC metabolomics studies, only three studies reported MELD scores for HCC and cirrhosis patients (14, 15, 18), and two studies indicated the Child-Pugh status of their HCC or cirrhosis cohorts (7, 18). The lack of information on whether liver function is compensated or decompensated complicates interpretation of metabolomic data. Body mass index (BMI), which also went unreported in all but one of the referenced HCC metabolomics studies (21), was delineated in our work. BMI can significantly influence metabolite expression levels, particularly with regard to adiposity. The lack of adequate matching of HCC patients and cirrhosis controls with regard to cirrhosis aetiology further complicates interpretation of the metabolomic expression patterns between the two groups. HCC emerges from viral hepatitis B or C-associated cirrhosis, alcoholic cirrhosis, non-alcoholic steatohepatitis, primary biliary

cirrhosis and non-alcoholic fatty liver disease. These distinct aetiologies each likely leave a unique metabolomic fingerprint in the afflicted patient, and the insufficient matching of HCC patients and DCs in the referenced HCC metabolomics studies may explain the incongruent metabolic trends reported among these works. Because HCV-cirrhosis is the leading cause of HCC-related mortality in the USA, and to control for aetiological variances in metabolite expression profiles, we confined our aetiological inclusion criteria to HCC and DC patients possessing only histopathologically proven HCV-cirrhosis. Further, in the referenced HCC metabolomics studies, only seven reported the clinically relevant metabolomic comparison of HCC vs. cirrhosis (6, 7, 9, 14-17). Because HCC is a complex heterogeneous disease that most often emerges in the setting of cirrhosis and is often accompanied by significant comorbidity, we hypothesize that the comparison of HCC vs. NHC is not as clinically relevant as the comparison between HCC vs. cirrhosis. Hence, we confined our search for pathways relevant to HCC by comparing the metabolomes of HCC and cirrhosis patients rather than comparing HCC vs. NHC metabolomes. The referenced HCC metabolomics works largely did not match HCC patients and cirrhosis controls by appropriate aetiology or severity of cirrhosis, tumour burden and performance status, which are well established independent prognostic factors. Our study addresses these limitations by matching HCC and DC patients according to histologically proven HCV-associated cirrhosis, age, gender, race, MELD, Child–Pugh score and BMI. The majority of our patients had a MELD score <10 and all had Child–Pugh A cirrhosis. Our patients were also abstinent from alcohol and illicit drugs, further controlling for potential confounders in the interpretation of pathways related to the presence of HCC.

A limitation of this study is that while we reported on the differences between HCC vs. DC, DC vs. NHC and HCC vs. NHC (Table 2a), we did not analyse the metabolomes of chronic HCV patients without cirrhosis. The primary objective of this work was to characterize the metabolic disturbances associated with the presence of HCC. Our DC vs. NHC comparison is relevant because of the clinically silent nature of chronic hepatitis infection, but a future comparison of HCV-only vs. HCV-cirrhosis will better illustrate the pathways driving of the development of cirrhosis and clarify whether the metabolic disturbances we identify as important to the presence of cirrhosis are exhibited in HCV-infected patients without cirrhosis. Another limitation in our study is that the sensitivity and specificity values in Table 3 were generated on the basis of each metabolite's mass spectrometry peak area. Absolute quantification using selected or multiple reaction monitoring mass spectrometry is a suitable strategy for accurate quantification of small molecules <1 kD. To further characterize whether the significantly altered metabolites in this work have a potential diagnostic value for HCC and/or cirrhosis, targeted metabolomics should be employed in other patient cohorts. Finally, studies integrating HCC metabolomic data with other 'omics' data have recently been conducted (21, 22) and these studies contribute to a more systems-based understanding of HCC. Coupling metabolomic data with genomic data can provide valuable and deeper insights into the molecular underpinnings of HCC and the efforts of Budhu *et al.* and Beyoglu and colleagues should be built upon in future HCC metabolomics studies.

In conclusion, integrated GC/MS and UPLC/MS-MS metabolomics identified multiple metabolic disturbances in HCC patients vs. HCV-cirrhosis controls. We also identified a possible strong metabolic signature of cirrhosis. Secondary investigations of these pathways' relationship with HCC and cirrhosis are merited.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the NIH KL2 University of Florida Clinical Translational Science Scholar Award (R.C.), NIH/NCRR award UL1RR029890 (D.R.N., R.C.) and NIH/NCI award K24CA139570 (D.R.N.). We thank Lauren McIntyre of the Southeast Center for Integrated Metabolomics (SECIM) for providing her statistical expertise. We also thank the participants of this study for their dedication and commitment.

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Fig. 1. Random forest (RF) supervised class prediction using patients' global metabolomic expression profiles. Mean decrease accuracy (MDA) denotes the per cent decrease in accuracy of the random forest analysis when the trial is performed in the absence of the indicated biomarker. (A) Utility of RF to accurately classify normal healthy controls (NHC), DC and HCC patients into their appropriate groups on the basis of their global metabolomic profiles. With all three groups included, 2-pyrrolidinone, a GABA metabolite, possessed a MDA of 8%, making it the most important metabolite for RF accuracy. (B) RF analysis of

HCC patients ($n = 30$) vs. disease control patients with HCV-associated cirrhosis (DC, $n = 27$). Several amino acids ranked among the most important metabolites for the RF class prediction accuracy of HCC vs. DC, with serine being the most important metabolite for this predictive accuracy.

Table 1

Clinical characteristics of hepatocellular carcinoma (HCC) ($n = 30$) and HCV-cirrhosis ($n = 27$) patients. Patients were matched by age, gender and body mass index (BMI). All patients had well-compensated Child–Pugh A cirrhosis and the majority of patients in both the HCC and the DC groups had a MELD score <10 . All patients had histopathologically proven chronic HCV-associated cirrhosis. To control for biological and lifestyle confounders, we recruited non-diabetic patients with no other cancers and who were abstinent from drug use and alcohol consumption. Normal healthy controls ($n = 30$, not shown) were matched by age and gender (male $n = 15$, female $n = 15$)

Factor	Category	HCC ($n = 30$) <i>N</i> , or mean (range)	DC ($n = 27$) <i>N</i> , or mean (range)
Age		59.9 (48–72)	55.4(31–72)
Gender	Male	19	12
	Female	11	15
Race	Caucasian	24	22
	African American	4	2
	Hispanic	1	2
	Asian	1	1
Aetiology	HCV-Cirrhosis	30	27
AFP	400 ng/ml	19	26
	>400 ng/ml	11	0
	No data	0	1
BMI	Male	24.6(17.6–31)	28.5 (17.7–15.0)
	Female	24.6 (20.4–30)	28.2(21.0–34.0)
Child–Pugh	A	30	27
MELD	<10	22	19
	10	8	8
HCC Stage *	A	13	N/A
	B	10	
	C	7	
Diabetic	Yes	0	0
	No	30	27

AFP, alpha-foetoprotein; MELD, Model for End-Stage Liver Disease.

* Stage based on the Barcelona clinic liver cancer staging system.

Table 2

Significantly altered metabolites in HCC and HCV -cirrhosis. Fold-difference values in bold are significant ($P < 0.05$). (a) Fifty-four metabolites significantly associated with the presence of HCC. Pathways of importance to HCC were as follows: Acylcarnitine metabolism, amino acid metabolism, eicosanoid lipid signalling cascades, dicarboxylic acid metabolism, glycerophospholipid metabolism, N-acetyl amino acids and oxidative stress homeostasis metabolites, (b) Thirty-eight metabolites significantly associated with the presence of cirrhosis. Dicarboxylic acids and bile acids were markedly elevated in HCV -cirrhosis vs. healthy volunteers, m/z , mass-to-charge ratio. RT, retention time.

(a)						
RT	m/z	Platform	Metabolic Pathway	Biochemical Name	HCC/DC Fold Difference	DC/NC Fold Difference
1401	262.1	LC/MS pos	Acylcarnitine metabolism - Medium chain	Succinylcarnitine	1.31	0.89
1203	204.2	LC/MS pos	Acetylarnitine metabolism - Medium chain	Acetylarnitine	1.2	1.01
1565	276.1	LC/MS pos	Acetylarnitine metabolism - Medium chain	Glutarylarnitine (C5)	1.28	0.86
1763.1	348.2	GC/MS	Amino acids	Aspartate	1.42	0.51
1836.8	362.2	GC/MS	Amino acids	Glutamate	1.9	0.51
1656.3	320.2	GC/MS	Amino acids	Serine	1.35	0.73
1486.9	218.1	GC/MS	Amino acids	Glycine	1.36	0.7
2056	166.1	LC/MS pos	Amino acids	Phenylalanine	1.22	0.81
1726.1	334.2	GC/MS	Amino acids	Homoserine	1.62	0.71
3349	352.2	LC/MS pos	Dipeptide	Phenylalanyltryptophan	0.84	0.97
1808	253.1	LC/MS pos	Dipeptide	Phenylalanylserine	0.8	2.55
1760	203.2	LC/MS pos	Dipeptide	Leucylalanine	0.76	2.24
5295	319.3	LC/MS neg	Eicosanoid lipid signalling Cascade	12-HETE	1.69	0.13
5271	319.3	LC/MS neg	Eicosanoid lipid signalling Cascade	15-HETE	2.8	0.34
5600	305.4	LC/MS neg	Eicosanoid lipid signalling Cascade	Dihomo-linolenate (20:3n3 or n6)	1.27	0.83
5450	277.3	LC/MS neg	Eicosanoid lipid signalling Cascade	Linolenate [alpha or gamma; (18:3n3 or 6)]	1.46	0.81
5533	279.3	LC/MS neg	Eicosanoid lipid signalling Cascade	Linoleate (18:2n6)	1.25	0.91
5722	307.3	LC/MS neg	Eicosanoid lipid signalling Cascade	Dihomo-linoleate (20:2n6)	1.41	1.13

(a)

RT	m/z	Platform	Metabolic Pathway	Biochemical Name	HCC/DC Fold Difference	HCC/DC P	DC/NC Fold Difference	DC/NC P
5525	303.4	LC/MS neg	Eicosanoid lipid signalling Cascade	Arachidonate (20:4n6)	1.23	0.0268	0.72	1.93E-05
5270	295.2	LC/MS neg	Eicosanoid lipid signalling Cascade	13-HODE + 9-HODE	2.54	0.0307	0.52	1.05E-06
1623	101.2	LC/MS neg	Fatty acid metabolism	Isovalerate	2.63	0.0038	0.69	0.1201
2071	131	GC/MS	Fatty acid, amide	Stearamide	1.38	0.0102	0.48	1.01 E-09
1322	187.2	LC/MS neg	Fatty acid, dicarboxylate	Azelate (nonanedioate)	1.77	0.0184	54.35	0.00E+00
1778	201.2	LC/MS neg	Fatty acid, dicarboxylate	Sebacate (decanedioate)	1.66	0.0298	25.78	0.00E+00
2376	215.1	LC/MS neg	Fatty acid, dicarboxylate	Undecanedioate	1.83	0.0331	36.09	0.00E+00
2990	229.2	LC/MS neg	Fatty acid, dicarboxylate	Dodecanedioate	1.54	0.0335	2.27	1.43E-06
2045	265.3	GC/MS	Fatty acid, ester	n-Butyl Oleate	1.43	0.012	1.03	0.3653
5508	271.3	LC/MS neg	Fatty acid, monohydroxy	2-Hydroxyypa Imitate	1.24	0.0011	1.37	7.76E-06
4681	187.2	LC/MS neg	Fatty acid, monohydroxy	3-Hydroxydecanoate	1.29	0.0149	1.14	0.0238
5705	299.4	LC/MS neg	Fatty acid, monohydroxy	2-Hydroxystearate	1.17	0.0191	1.24	0.0032
4826	187.2	LC/MS neg	Fatty acid, monohydroxy	2-Hydroxydecanoic acid	1.87	0.0286	1.24	0.4604
2323	159.1	LC/MS neg	Fatty acid, monohydroxy	8-Hydroxyoctanoate	1.45	0.0371	3.31	3.48E-12
1190.9	142	GC/MS	GABA Metabolism	2-Pyrrolidinone	1.27	0.0082	3.59	0.00E+00
5752	409.3	LC/MS neg	Glycerolipid metabolism	1-Palmitoylglycerophosphate	1.58	0.006	0.61	9.11 E-06
5848	478.3	LC/MS neg	Glycerolipid metabolism	2-Oleoylglycerophosphoethanolamine	1.78	0.0074	0.57	0.023
5940	452.3	LC/MS neg	Glycerolipid metabolism	1-Palmitoylglycerophosphoethanolamine	1.53	0.0268	0.62	0.045
5790	452.3	LC/MS neg	Glycerolipid metabolism	2-Palmitoylglycerophosphoethanolamine	1.24	0.0284	0.66	0.012
5928	478.3	LC/MS neg	Glycerolipid metabolism	1-Oleoylglycerophosphoethanolamine	2.07	0.0384	0.49	0.0078
674	104.2	LC/MS pos	Glycerolipid metabolism	Choline	1.17	0.0074	0.64	4.20E-12
882	130.1	LC/MS neg	N-Acetyl amino acids	N-acetylalanine	1.21	0.0012	0.89	0.0002
1396.4	143.9	GC/MS	N-Acetyl amino acids	N-acetylglycine	1.68	1.13E-05	0.74	1.22E-11
1526	218	GC/MS	N-Acetyl amino acids	N-acetyls erine	1.26	0.0001	0.96	1.43E-05
1134	189.1	LC/MS pos	N-Acetyl amino acids	N6-acetyll ysine	1.18	0.0017	0.86	6.39E-09
2846	295.1	LC/MS pos	Oxidative stress homeostasis	Gamma-glutamylphenylalanine	1.4	0.0013	0.9	0.0576
2644	261.2	LC/MS pos	Oxidative stress homeostasis	Gamma-glutamylisoleucine	1.59	0.0057	0.84	0.0599
977	249.1	LC/MS pos	Oxidative stress homeostasis	Gamma-glutamylthreonine	1.43	0.0139	0.91	0.1827
2040	247.2	LC/MS pos	Oxidative stress homeostasis	Gamma-glutamylvaline	1.62	0.034	0.76	0.0004

(a)							
RT	m/z	Platform	Metabolic Pathway	Biochemical Name	HCC/DC Fold Difference	HCC/DC P	DC/NC P
1169.4	130.9	GC/MS	Oxidative stress homeostasis	2-Hydroxybutyrate (AHB)	1.56	0.0003	0.83
1889.9	353	GC/MS	i.e. metabolism, (hypo)xanthine/inosine contai	Xanthine	1.62	9.35E-05	0.51
1313	135.1	LC/MS neg	i.e. metabolism, (hypo)xanthine/inosine contai	Hypoxanthine	1.24	0.0346	0.2
1356	282.1	LC/MS pos	Purine metabolism, adenine containing	N1-Methyladenosine	1.17	0.0089	1.25
1804.2	264	GC/MS	Purine metabolism, adenine containing	Adenine	1.91	0.0371	2.24
1430	243.1	LC/MS neg	Pyrimidine metabolism, uracil containing	Uridine	1.2	0.0035	0.72
5197	300.2	LC/MS pos	Sphingolipid	Sphingosine	1.87	0.0017	0.44
(b)							
RT	m/z	Platform	Pathway	Biochemical Name	DC/NC Fold Difference	P	HCC/DC Fold Difference
5218	498.3	LC/MS neg	Bile acid metabolism	Taurochenodeoxycholate (TCDCA)	17.78	5.74E-07	16.09
5122	514.3	LC/MS neg	Bile acid metabolism	Taurocholate (TCA)	14.97	1.37E-08	17.96
4762	279.8	LC/MS neg	Bile acid metabolism	Taurochenolate sulfate*	6.54	1.12E-08	8
5002	562.1	LC/MS neg	Bile acid metabolism	Taurolithocholate 3-sulfate	5.15	0.0011	4.82
5104	464.4	LC/MS neg	Bile acid metabolism	Glycocholate (GCA)	4.18	1.28E-06	6.18
5002.6	498.4	LC/MS neg	Bile acid metabolism	Tauroursodeoxycholate (TDCA)	4.11	1.44E-05	4.41
5245	448.4	LC/MS neg	Bile acid metabolism	Glycochenodeoxycholate (GDCA)	3.24	0.0002	3.68
4100	288.3	LC/MS pos	Acylcarnitine metabolism - Medium chain	Octanoylcarnitine	1.98	0.0075	1.76
4700	316.3	LC/MS pos	Acylcarnitine metabolism - Medium chain	Decanoylcarnitine	1.99	0.0274	1.37
5066	344.3	LC/MS pos	Acylcarnitine metabolism - Medium chain	Laury carnitine	2.19	0.0002	2.03
1941	232.2	LC/MS pos	Acylcarnitine metabolism - Long chain	Isobutyrylcarnitine	0.49	7.24E-06	0.58
5202	426.4	LC/MS pos	Acylcarnitine metabolism - Long chain	Oleoylcarnitine	0.75	0.0129	1.03
5179	400.4	LC/MS pos	Acylcarnitine metabolism - Long chain	Palmitoylcarnitine	0.82	0.03	1.09

(b)

RT	m/z	Platform	Pathway	Biochemical Name	DC/NC Fold Difference	P	HCC/NC Fold Difference	P	HCC/DC Fold Difference	P
1322	187.2	LC/MS neg	Fatty acid, dicarboxylate	Azelate (nonanedioate)	54.35	0.00E+00	96.08	0.00E+00	1.77	0.0184
2376	215.1	LC/MS neg	Fatty acid, dicarboxylate	Undecanedioate	36.09	0.00E+00	66.05	0.00E+00	1.83	0.0331
1778	201.2	LC/MS neg	Fatty acid, dicarboxylate	Sebacate (decanedioate)	25.78	0.00E+00	42.91	0.00E+00	1.66	0.0298
4650	285.2	LC/MS neg	Fatty acid, dicarboxylate	Hexadecanedioate	6.08	1.60E-14	6.56	0.00E+00	1.29	-
3970	257.2	LC/MS neg	Fatty acid, dicarboxylate	Tetradecanedioate	2.65	1.80E-05	2.7	1.06E-07	1.02	-
4650	285.2	LC/MS neg	Fatty acid, dicarboxylate	Hexadecanedioate	5.08	1.60E-14	6.56	0.00E+00	1.29	-
5206	197.2	LC/MS neg	Medium chain fatty acid	5-dodecenoate (12:1 n7)	2.88	0.0004	3.78	6.61 E-08	1.31	-
5086	183.3	LC/MS neg	Medium chain fatty acid	10-undecenoate (11:1 n1)	2.03	1.76E-06	2.1	2.30E-09	1.04	-
5288	199.3	LC/MS neg	Medium chain fatty acid	Laurate (12:0)	1.65	0.0002	2.18	5.60E-06	1.32	-
4847	157.2	LC/MS neg	Medium chain fatty acid	Pelargonate (9:0)	1.62	1.86E-09	1.72	3.38E-07	1.06	-
3695	129.2	LC/MS neg	Medium chain fatty acid	Heptanoate (7:0)	1.55	1.68E-07	1.59	6.22E-07	1.02	-
4367	143.2	LC/MS neg	Medium chain fatty acid	Caprylate (8:0)	1.46	4.05E-05	1.6	1.42E-05	1.11	-
5092	171.2	LC/MS neg	Medium chain fatty acid	Caprate (10:0)	1.45	0.0006	1.77	1.14E-05	1.22	-
5207	185.2	LC/MS neg	Medium chain fatty acid	Undecanoate (11:0)	1.33	1.49E-06	1.48	1.06E-09	1.12	-
2766	115.2	LC/MS neg	Medium chain fatty acid	Caproate (6:0)	1.29	0.0004	1.37	3.03E-06	1.07	-
3379	733.5	LC/MS pos	Fibrinogen cleavage peptide	DSGEGDFXAEGGGVVR*	11.81	1.04E-06	4.08	1.07E-05	0.35	0.0462
3239	769	LC/MS pos	Fibrinogen cleavage peptide	ADSGEGDFXAEGGGVVR*	2.92	1.63E-12	3.26	7.72E-07	1.12	-
4812	583.2	LC/MS neg	Hemoglobin and porphyrin metabolism	Bilirubin (Z,Z)	4.01	7.98E-07	3.97	9.36E-08	0.99	-
4671	581.2	LC/MS neg	Hemoglobin and porphyrin metabolism	Biliverdin	1.97	0.0087	2.33	6.08E-05	1.18	-
1041	117	GC/MS	Ketogenesis	1,2-propanediol	5.77	5.00E-15	6.88	1.00E-15	1.19	-
855	185.1	LC/MS neg	Dipeptide	Pyroglutamylglycine	0.53	6.46E-07	0.79	0.007	1.48	0.0136
1572	175.1	LC/MS pos	Dipeptide	Glycylvaline	0.44	7.00E-06	0.85	0.0311	1.95	0.0072
1379.5	271.1	GC/MS	Dipeptide	Alanylalanine	0.35	6.45E-10	0.35	2.22E-10	0.99	-
2310	247.2	LC/MS pos	Dipeptide	Aspartyl leucine	0.32	2.04E-10	0.38	6.30E-10	1.18	-
1805	272.2	LC/MS neg	Dipeptide	Aspartyl leucine	3.58	1.32E-05	2.99	5.13E-06	0.84	-

Table 3

Receiver operator characteristic (ROC) analysis of significantly altered metabolites. Metabolites investigated through ROC analysis were selected on the basis of their value to random forest, *P*-value and false discovery rate, and fold difference in cases vs. controls. Mass spectrometry peak areas corresponding to expression level in each patient were used in the ROC analysis. (a) Metabolites significantly associated with the presence of HCC. (b) Predictive utility of metabolites significantly associated with the presence of cirrhosis. Area under the curve (AUC) and sensitivity/specificity/fold difference in the DC vs. NHC comparison were more patent than those of the HCC vs. DC comparison.

(a)						
Pathway	Metabolite	Sensitivity	Specificity	AUC	<i>P</i>	Fold difference in HCC vs. DC
12-lipoxygenase, cytochrome P450	12-HETE	73.3	69.2	0.729	0.0011	1.69 up
15-lipoxygenase, cytochrome P450	15-HETE	83.3	59.3	0.705	0.0041	2.80 up
Endothelial celladhesion; inflammation	13-HODE + 9-HODE	73.3	66.7	0.678	0.0159	2.54 up
Leucine and valine degradation	Isovalerate	60.0	81.5	0.734	0.0005	2.63 up
Amino acid	Aspartate	100.0	51.9	0.789	<0.0001	1.42 up
	Glycine	83.3	63.0	0.801	<0.0001	1.36 up
	Serine	73.3	85.2	0.831	<0.0001	1.35 up
	Phenylalanine	73.3	81.5	0.780	<0.0001	1.22 up
	Homoserine	70.0	85.2	0.765	0.0001	1.62 up
Acid ceramidase	Sphingosine	58.3	86.7	0.731	0.0058	1.87 up
Nucleotide catabolism; ROS	Xanthine	63.3	88.9	0.790	<0.0001	1.62 up
ROS neutralization	2-Hydroxybutyrate	76.7	77.8	0.777	<0.0001	1.56 up

(b)						
Pathway	Metabolite	Sensitivity	Specificity	AUC	<i>P</i>	Fold difference in DC vs. NHC
Dicarboxylic acids	Azelate	100	100	1.00	0.00	54.4 up
	Sebacate	100	100	1.00	<0.0001	25.8 up
	Undecanedioate	77.8	90.0	0.860	<0.0001	36.1 up
	2-hydroxyglutarate	92.6	90.0	0.965	<0.0001	2.5 up
	Hexadecanedioate	100	93.3	0.967	<0.0001	5.1 up
Bile acid metabolism	Taurochenodeoxycholate (TCDCA)	77.8	90.0	0.860	<0.0001	17.8 up
	Taurocholate (TCA)	85.2	80.0	0.898	<0.0001	15.0 up
	Taurocholate sulphate	74.1	100.0	0.932	<0.0001	6.5up
	Glycohyocholate (GHCA)	85.2	83.3	0.912	<0.0001	6.6up
	Glycocholate(GCA)	77.8	90.0	0.852	<0.0001	4.2 up
	Tauroursodeoxycholate	81.5	76.5	0.831	<0.0001	4.1up
	Glycochenodeoxycholate (GCDCA)	74.1	86.7	0.779	<0.0001	3.2up
	Taurolithocholate 3-sulphate	63.0	83.3	0.747	<0.0003	5.2 up
	Phenethylamine	77.8	96.7	0.873	<0.0001	14.2 up
Ketogenesis	1 2-propanediol	96.3	96.7	0.964	<0.0001	5.8 up
Steroid	Androsterol monosulphate 2	74.1	96.7	0.910	<0.0001	5.8 up
Fibrinogen cleavage peptides	DSGEGDFXAEGGGVR	81.5	90.0	0.920	<0.0001	11.8 up

(b)

Pathway	Metabolite	Sensitivity	Specificity	AUC	P	Fold difference in DC vs. NHC
	ADSGEGDFXAEggGVR	88.9	90.0	0.953	<0.0001	2.9 up
GABA metabolism	2-Pyrrolidmone	100	100	1.00	0.00	3.6 up
Haeme metabolism	Bilirubin (z, z)	85.2	90.0	0.911	<0.0001	4.0 up
	Urobilinogen	92.6	93.3	0.958	<0.0001	11.5 up
Glycerophospholipid metabolism	1-stearoylqlycerophosphocholine	85.2	86.7	0.922	<0.0001	0.34 down