

## Disease monitoring of hepatocellular carcinoma through metabolomics

Asem I Fitian, Roniel Cabrera

Asem I Fitian, School of Medicine, Virginia Commonwealth University, Richmond, VA 23298, United States

Roniel Cabrera, Department of Medicine, Division of Gastroenterology, Hepatology and Nutrition, Section of Hepatobiliary Diseases, University of Florida, Gainesville, FL 32610, United States

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**Correspondence to:** Roniel Cabrera, MD, MS, Department of Medicine, Division of Gastroenterology, Hepatology and Nutrition, Section of Hepatobiliary Diseases, University of Florida, 1600 SW Archer Rd M440, Gainesville, FL 32610, United States. [roniel.cabrera@medicine.ufl.edu](mailto:roniel.cabrera@medicine.ufl.edu)  
**Telephone:** +1-352-2739500  
**Fax:** +1-352-3927393

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### Abstract

We elucidate major pathways of hepatocarcinogenesis and accurate diagnostic metabolomic biomarkers of hepatocellular carcinoma (HCC) identified by contemporary HCC metabolomics studies, and delineate a model HCC metabolomics study design. A literature search was carried out on Pubmed for HCC metabolomics articles published in English. All relevant articles were accessed in full text. Major search terms included "HCC", "metabolomics", "metabolomics", "metabonomic" and "biomarkers". We extracted clinical and demographic data on all patients and consolidated the lead candidate biomarkers, pathways, and diagnostic performance of metabolomic expression patterns reported by all studies in tables. Where reported, we also extracted and summarized the metabolites and pathways most highly associated with the development of cirrhosis in table format. Pathways of lysophospholipid, sphingolipid, bile acid, amino acid, and reactive oxygen species metabolism were most consistently associated with HCC in the cited works. Several studies also elucidate metabolic alterations strongly associated with cirrhosis, with  $\gamma$ -glutamyl peptides, bile acids, and dicarboxylic acids exhibiting the highest capacity for stratifying cirrhosis patients from appropriately matched controls. Collectively, global metabolomic profiles of the referenced works exhibit a promising diagnostic capacity for HCC at a capacity greater than that of conventional diagnostic biomarker alpha-fetoprotein. Metabolomics is a powerful strategy for identifying global metabolic signatures that exhibit potential to be leveraged toward the screening, diagnosis, and management of HCC. A streamlined study design and patient matching methodology may improve concordance among metabolomic datasets in future works.

**Key words:** Metabolomics; Hepatocellular carcinoma; Biomarkers; Metabolic profiling; Chromatography/mass spectrometry; Noninvasive biomarkers; Cirrhosis

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**Core tip:** The high-throughput, validated nature of metabolomics makes it an ideal methodology for rapidly identifying the global metabolic alterations associated with hepatocarcinogenesis - alterations that not only enhance our understanding of the metabolic underpinnings of cirrhosis and hepatocellular carcinoma (HCC), but that can be leveraged to improve HCC diagnostic, therapeutic, and disease monitoring efficacy. Indeed, contemporary HCC metabolomics works time and again demonstrate this promise that metabolomics platforms hold in serving as standalone non-invasive HCC diagnostic and disease monitoring modalities.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the world's third most lethal cancer, possessing a five-year survival rate of 10% that results in between 250000 to 1000000 deaths per year<sup>[1,2]</sup>. HCC culminates from a preexisting long-term condition of cirrhosis in 90% of cases<sup>[3]</sup> and cirrhosis patients are among the best characterized individuals at high risk for developing cancer. Notwithstanding the opportunity for clinical surveillance of these patients, the dismal survival rate persists and HCC has now emerged as the fastest rising cause of cancer related death in the United States<sup>[4,5]</sup>. HCC patients in Asia and sub-Saharan Africa face a particularly grim outlook, with > 90% of patients in rural areas of these regions progressing within the first year of HCC onset<sup>[6]</sup>. A major hindrance to successful early diagnosis of HCC stems from the substandard accuracy of the principal HCC diagnostic modalities. Alpha-fetoprotein (AFP) is the principal biomarker for HCC and despite the inexpensive and reproducible nature of the AFP blood test, its sensitivity of 25%-65%<sup>[7,8]</sup> for HCC has exacerbated early detection of this cancer. The low sensitivity of AFP can be explained by the fact that up to 40% of HCC and cirrhosis patients have normal AFP levels, and that AFP is often elevated in patients without HCC<sup>[9-11]</sup>. Moreover, only 10%-20% of patients with early-stage HCC have elevated AFP levels<sup>[12]</sup>. Exclusion of AFP as an HCC diagnostic modality in the AASLD guidelines for HCC surveillance underscores AFP's unreliability to accurately screen for early HCC<sup>[13]</sup>. Efforts to overcome this substandard performance have resulted in the identification and commercialization of novel HCC biomarkers des-gamma-carboxyprothrombin and lectin-bound AFP (AFP-L3). These markers are ineffective when used alone as HCC biomarkers, however,

and even when combined with AFP still demonstrate poor sensitivity for HCC, particularly in the detection of lesions < 3 cm<sup>[14]</sup>. While magnetic resonance imaging and computed tomography offer better accuracy in HCC diagnosis, these sophisticated diagnostic modalities are both economically and logistically incompatible with the resource-poor areas experiencing the brunt of HCC's mortality rate<sup>[15-17]</sup>. Improving early HCC detection and patient outcome globally requires fulfilling of the urgent need for a reproducible, inexpensive, and accurate HCC diagnostic test.

### **Metabolomics as an HCC biomarker discovery tool**

Numerous genomic and proteomic screening studies have been employed to identify potential biomarkers of HCC<sup>[18-24]</sup> but to date the markers identified in these studies have not been clinically fruitful. Because the liver is the hub of carbohydrate, amino acid, and lipid metabolism<sup>[25,26]</sup>, chronic liver diseases undoubtedly disrupt normal metabolic function. A metabolomics analysis of HCC and cirrhotic tissue can therefore elucidate not just the metabolic pathways most relevant to the hepatocarcinogenic process, thereby identifying metabolites showing promise as HCC biomarkers, but also global metabolic patterns that serve as comprehensive disease signatures and which may therefore be used to stratify cases from controls. Metabolomics is the comprehensive identification of all small metabolites < 2 kD in a tissue sample. Through combined gas or liquid chromatography/mass spectrometry, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI TOF-MS) or nuclear magnetic resonance instrumentation, metabolomics platforms enable investigators to rapidly screen hundreds of metabolites in a large series of biofluid or solid tissue samples and are capable of simultaneously detecting metabolites belonging to a diverse array of pathways including amino acids, lipids, carbohydrates, and nucleotides. Metabolomics platforms are translationally optimal and hold potential for clinical implementation because they reveal global metabolite expression pattern differences among cases and controls in an automated, rapid, high-throughput, quality controlled, and reproducible manner. Metabolomics facilitates rapid identification of diagnostic markers, prognostic markers, and lead drug target pathways and their implementation in drug discovery divisions of pharmaceutical giants underscores their important role in the lead target generation realm<sup>[27]</sup>.

Nearly two dozen HCC metabolomics studies have been reported<sup>[28-46]</sup>. These studies illustrate the key pathways involved in stepwise hepatocarcinogenesis and reveal metabolites that may have utility as biomarkers of HCC and cirrhosis. The findings of these studies are summarized in Tables 1 and 2. These works reveal deregulation of bile acid metabolism, fatty acid  $\beta$ -oxidation by way of the carnitine palmitoyltransferase (CPT) shuttle system, amino acid metabolism, and glycerophospholipid metabolism in HCC vs cirrhosis,

**Table 1** Significantly altered metabolites in hepatocellular carcinoma patients *vs* cirrhosis controls

Ref.	Platform	Tissue (organism)	HCC etiology	Significantly altered metabolites in HCC patients <i>vs</i> cirrhosis controls	Main pathways distinguishing HCC from cirrhosis
Patterson <i>et al</i> <sup>[29]</sup>	UPLC/QTOF-MS; UPLC/ESI-TQMS; GC/MS	Plasma (human)	HCC <i>n</i> = 20: EtOH <i>n</i> = 6 HBV <i>n</i> = 3 HCV <i>n</i> = 5 NASH <i>n</i> = 3 NASH/alcoholic steatohepatitis <i>n</i> = 1 HH <i>n</i> = 2	LPC (14:0) ↓ LPC (18:1) ↓ LPC (20:4) ↓ LPC (20:3) ↓ LPC (22:6) ↓ FFA (24:0) - lignoceric acid ↓ FFA (24:1) - nervonic acid ↓ Bilirubin ↑ Biliverdin ↑	LPC metabolism Very long chain fatty acid metabolism Hemoglobin metabolism
Xiao <i>et al</i> <sup>[37]</sup>	UPLC/QTOF-MS	Serum (human)	HCV	3β, 6β-dihydroxy-5β-cholan-24-oic acid ↓ 3α, 7β-dihydroxy-5β-cholest-24-en-26-oic acid ↓ GCA ↓ GDCA ↓ GCDCA ↓ TCDCa ↓ Linoelaidyl carnitine ↓ Oleoylcarnitine ↓ Palmitoyl carnitine ↓ O-octanoyl-R-carnitine ↓ LPC (20:1) ↓ LPC (20:4) ↓ PE (20:4/18:1) ↓ 4E;15Z-Bilirubin IXa ↓ 15,16-dihydrobiliverdin ↓ 3-ganidinopropionic acid ↓ Tetracosahexaenoic acid ↓ 3-hydroxy-eicosanoic acid ↓ Oleamide ↓ Phe-Phe ↑	Bile acid biosynthesis CPT shuttle system LPC metabolism Hemoglobin metabolism Eicosanoid metabolism
Wang <i>et al</i> <sup>[38]</sup>	UPLC/MS-MS; LC/QTOF-MS	Serum (human)	HCC <i>n</i> = 82: HBV-cirrhosis <i>n</i> = 41 HBV-only <i>n</i> = 41	LPC-16:0 ↑ LPC-18:0 ↑ 16:0/18:1-PC ↑ 16:0/18:2-PC ↑ 16:0/20:4-PC ↑ 16:0/22:6-PC ↑ 18:0/18:2-PC ↑ Phenylalanine ↓ GCDCA ↓ Canavaninosuccinate ↑↑	LPC metabolism Gut flora metabolism Bile acid metabolism Organic acid metabolism
Zhou <i>et al</i> <sup>[39]</sup>	UPLC/QTOF-MS	Serum (human)	HCC <i>n</i> = 69: HBV-HCC (HCC-B) <i>n</i> = 38 HCV-HCC (HCC-C) <i>n</i> = 31	LPC (14:0) HCC-C ↓ LPC (16:1) HCC-C ↓ LPC (18:3) HCC-B ↓ HCC-C ↓ LPC (18:2) HCC-B ↓ HCC-C ↓ LPC (18:1) HCC-B ↓ HCC-C ↓ LPC (18:0) HCC-C ↓ LPC (20:5) HCC-C ↓ LPC (20:4) HCC-B ↓ HCC-C ↓ LPC (20:3) HCC-B ↓ HCC-C ↓ LPC (20:2) HCC-C ↓ LPC (20:0) HCC-B ↓ HCC-C ↓ LPC (22:6) HCC-C ↓ LPC (22:5) HCC-B ↓ HCC-C ↓ LPC (22:4) HCC-B ↓ HCC-C ↓	LPC metabolism
Wu <i>et al</i> <sup>[31]</sup>	SELDI-TOF-MS; HPLC/MS	Serum (human)	HBV	GRO-α ↑ Thrombin light chain ↑	Cytokine Protease cleavage
Ressom <i>et al</i> <sup>[36]</sup>	UPLC/QTOF-MS	Serum (human)	HCC <i>n</i> = 78: → HCV 67%	GDCA ↓ TCA ↓	Bile acid metabolism

			→ HBV 15% → Alcoholism 29% → NASH 13% → Cryptogenic (8%) → Autoimmune (3%)	TCDCA ↓ Sphingosine 1-phosphate ↑ LPC (16:0) ↑ LPC (17:0) ↑ LPC (18:0) ↑ LPC (15:0) ↑ LPC (22:6) ↑ LPE (22:6) ↑ LPE (20:4) ↑ LPE (20:3) ↑ PS ↑ Glucose ↓ Creatine ↓ PE ↑ Glutamine ↑ Glutamate ↑ PC + GPC ↑	Sphingolipid metabolism LPC metabolism
Yang <i>et al</i> <sup>[28]</sup>	HRMAS 1H NMR	Biopsy (human)	HCC <i>n</i> = 17: → Cirrhosis <i>n</i> = 9 → No cirrhosis <i>n</i> = 8		Glycolysis LPC metabolism Amino acid metabolism
Nahon <i>et al</i> <sup>[40]</sup>	NMR	Serum (human)	EtOH cirrhosis	High density lipoproteins Acetate ↑ N-acetyl-glycoproteins ↑ Glutamate ↑ Glutamine ↓	Bile acid metabolism HDL biosynthesis Ketone body metabolism N-acetyl-glycoprotein Amino acid metabolism
Budhu <i>et al</i> <sup>[43]</sup>	GC/MS, UPLC/MS-MS	Biopsy samples (human)	HCC <i>n</i> = 356 Training cohort <i>n</i> = 30 Testing cohort <i>n</i> = 217 Validation cohort <i>n</i> = 139	Study reported on markers involved in cancer aggressivity through comparison of stem-like HCC to less benign mature hepatocyte HCC	N/A
Beyoğlu <i>et al</i> <sup>[44]</sup>	GC/MS	Biopsy samples (human)	Six HCC subtypes, liver fibrosis status unknown	Glucose ↓	Glycolysis
Fitian <i>et al</i> <sup>[45]</sup>	UPLC/MS-MS and GC/MS	Serum (human)	HCV cirrhosis-associated HCC <i>n</i> = 30 HCV-cirrhosis <i>n</i> = 27 Healthy volunteers <i>n</i> = 30	Glycerol 3-phosphate ↓ Glycerol 2-phosphate ↓ Malate ↓ Alanine ↓ Myo-inositol ↓ Linoleic acid ↓ Sphingosine ↑ Xanthine ↑ 2-Pyrrolidinone ↑ 2-Hydroxybutyrate ↑ Serine ↑ Glycine ↑ Aspartate ↑ 12-HETE ↑ 15-HETE ↑ Isovalerate ↑ Dihomo-linolenate ↑ Stearic acid	PI3K pathway Prostaglandin biosynthesis Sphingolipid Oxidative stress metabolism GABA metabolism Oxidative stress metabolism Amino acid Inflammation pathway Gut microflora metabolism Inflammation pathway Fatty acid biosynthesis
Gao <i>et al</i> <sup>[46]</sup>	GC-TOF/MS	Serum (human)	HBV cirrhosis-associated HCC <i>n</i> = 39 HBV-cirrhosis ( <i>n</i> = 52)	Heptadecanoic acid Palmitic acid 5-Aminovaleric acid Cholesterol ↑ 3-hydroxybutyric acid ↑ Malic acid ↑ Glutamine ↑ Asparagine ↓ Alanine ↑ Threonine ↓ Leucine ↓ Glutamic acid ↑ β-glutamate ↑ 5-oxoproline ↓ 1,2,4-cyclopropanodicarboxylic acid ↓	Gut microflora metabolism Cholesterol metabolism Ketogenesis TCA metabolism Amino acid Glutathione metabolism Dicarboxylic acid metabolism

Pathways of importance in the comparison of (1A) HCC *vs* cirrhosis and (2) cirrhosis *vs* healthy controls are shown. Arrows indicate the metabolite's expression in cases *vs* appropriate controls. *P* < 0.05 was used as the significance level and metabolites reported in table are those which were most significantly upregulated or downregulated in each study. EtOH: Alcohol; TOCSY: Total correlation spectroscopy; HH: Hereditary hemochromatosis; TCA: Tricarboxylic acid; UPLC: Ultrahigh-performance liquid chromatography; QTOF: Quadrupole time of flight; SELDI: Surface-enhanced laser desorption/ionization; HRMAS: High-resolution magic angle spinning; LPE: Lysophosphatidylethanolamine; LPC: Lysophosphatidylcholine; HCC: Hepatocellular carcinoma; MS: Mass spectrometry; TOF: Time-of-flight; GC: Gas chromatography; LC: Liquid chromatography; NMR: Nuclear magnetic resonance; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis; FFA: Free fatty acids; PE: Phosphorylethanolamine; GABA: γ-aminobutyric acid.

Table 2 Significantly altered metabolites in cirrhosis patients *vs* healthy volunteers

Ref.	Platform	Tissue (organism)	Significantly altered metabolites in cirrhosis patients <i>vs</i> healthy volunteers	Main pathways distinguishing cirrhosis from healthy volunteers
Gao <i>et al</i> <sup>[33]</sup>	<sup>1</sup> H NMR	Serum (human)	Isoleucine ↓ Leucine ↓ Valine ↓ Glutamine ↑ Tyrosine ↑ Phenylalanine ↑ 1-methylhistidine ↑ N-acetylglycoproteins ↑ Acetate ↑ Acetoacetate ↓ Pyruvate ↑ $\alpha$ -ketoglutarate ↑ Choline ↓ Taurine ↑ Glycerol ↑	Amino acid metabolism  N-acetylglycoprotein Ketonogenesis  Glycolysis TCA cycle  Bile acid metabolism Amino acid metabolism
Li <i>et al</i> <sup>[42]</sup>	UPLC/QTOF-MS	Serum (mouse)	Leucine ↓ Phenylpyruvic acid ↓ Phenylalanine ↓ Tryptophan ↓ LPE (16:0) ↓ LPE (18:0) ↓ LPC (16:0) ↓ LPC (20:1) ↓ LPC (22:6) ↑ PC (16:0/18:3) ↑ PC (12:1/24:3) ↑ PC (16:0/20:4) ↑ PC (16:0/22:6) ↑ PC (18:0/20:4) ↑ SM (d18:0/16:1) ↓	LPE metabolism  LPC metabolism  Phosphatidylcholine metabolism  Sphingomyelin metabolism
Soga <i>et al</i> <sup>[35]</sup>	Capillary electrophoresis/TOF-MS	Serum (human)	$\gamma$ -glutamylalanine ↑ $\gamma$ -glutamylvaline ↑ $\gamma$ -glutamylglutamine ↑ $\gamma$ -glutamylphenyl- $\gamma$ -glutamylcitrulline ↑ Alanine ↑ Methionine sulfoxide ↑	Glutathione metabolism  Amino acid metabolism
Wang <i>et al</i> <sup>[38]</sup>	UPLC/MS-MS; LC/QTOF-MS	Serum (human)	LPC-16:0 ↓ LPC-18:0 ↓ 16:0/18:1-PC ↓ 16:0/18:2-PC ↓ 16:0/20:4-PC ↓ 16:0/22:6-PC ↓ 18:0/18:2-PC ↓ Oleamide ↑ Phenylalanine ↑ GCDCA ↑ Canavaninosuccinate ↓	LPC metabolism  Fatty acid metabolism  Bile acid metabolism Arginosuccinate synthetase pathway
Zhou <i>et al</i> <sup>[39]</sup>	UPLC/QTOF-MS	Serum (human)	Phenylalanine ↑ GCA ↑ GDCA ↑ Bilirubin ↑ LPE (18:2) ↓ LPC (22:6) ↓ LPC (18:2) ↓ LPC (20:4) ↓ LPC (16:0) ↓ LPC (18:0) ↓ C18:1-CN ↑	Amino acid metabolism Bile acid metabolism  Hemoglobin metabolism Lysolipid metabolism
Chen <i>et al</i> <sup>[30]</sup>	UPLC/QTOF-MS	Serum (human); Urine (human)	Inositol ↓ 2,2-bipyridine ↓ Methionine ↓ Tyrosine ↓ Arginine ↓ Stearic acid ↓ Palmitic acid ↓ Citric acid ↓	CPT shuttle system TCA cycle  Amino acid metabolism  Fatty acid metabolism

Cao <i>et al</i> <sup>[32]</sup>	UPLC/MS	Fecal (human)	2-piperidine carboxylic acid ↓ 5-Hydroxy-tryptophan ↓ Chenodeoxycholic Acid dimeride ↓ Urobilin ↓ Urobilinogen ↓ 7-ketolithocholic acid ↓ LPC C18:0 ↑ LPC C16:0 ↑	Bile acid metabolism Hemoglobin metabolism Microbiome metabolism LPC metabolism
Yin <i>et al</i> <sup>[41]</sup>	RPLC/MS	Serum (human)	Hypoxanthine ↓ Inosine ↓ Bilirubin ↑ GCA ↑ GCDCA ↑ Taurine ↓ LPC C18:2 ↓ LPC C18:3 ↓ LPC C16:1 ↓ LPC C18:0 ↓ LPC C16:1 ↓ L-acetylcarnitine ↑	Purine synthesis Hemoglobin metabolism Bile acid metabolism LPC metabolism CPT shuttle system Nicotine metabolism
Fitian <i>et al</i> <sup>[45]</sup>	Integrated UPLC/MS-MS and GC/MS	Serum (human)	Glycocholate (GCA) ↑ Tauroursodeoxycholate ↑ Glychochemodeoxycholate ↑ Azelate (nonanedioate) ↑ Undecanedioate ↑ Sebacate (decanedioate) ↑ Hexadecanedioate ↑ Tetradecanedioate ↑ DSGEGDFXAEGGGVR ↑ ADSGEGDFXAEGGGVR ↑ Bilirubin (Z,Z) ↑ Biliverdin ↑ 1,2-propanediol ↑ Succinylcarnitine ↑ Acetylcarnitine ↑ Glutaryl carnitine ↑	Bile acid metabolism Dicarboxylic acid metabolism Fibrinogen cleavage peptide Hemoglobin catabolism metabolite Ketogenesis CPT shuttle system
Gao <i>et al</i> <sup>[46]</sup>	GC-TOF/MS	Serum (human)	Palmitic acid ↑ Stearic acid ↑ Oleic acid ↑ Arachidic acid ↑ Aminomalonic acid ↑ Phenylalanine ↑ Cysteine ↑ Leucine ↑ Citric acid ↑ Oxoproline ↑	Fatty acid metabolism Arachidonic acid metabolism Dicarboxylic acid metabolism Amino acid metabolism

EtOH: Alcohol; TOCSY: Total correlation spectroscopy; HH: Hereditary hemochromatosis; TCA: Tricarboxylic acid; UPLC: Ultrahigh-performance liquid chromatography; QTOF: Quadrupole time of flight; SELDI: Surface-enhanced laser desorption/ionization; HRMAS: High-resolution magic angle spinning; LPE: Lysophosphatidylethanolamine; LPC: Lysophosphatidylcholine; MS: Mass spectrometry; TOF: Time-of-flight; NMR: Nuclear magnetic resonance; LC: Liquid chromatography; GC: Gas chromatography; CPT: Carnitine palmitoyltransferase; TCA: Tricarboxylic acid.

and further delineate metabolites with potential utility in stratifying patients with cirrhosis from the healthy population.

### Pathways of importance in hepatocarcinogenesis

**Glycerophospholipids:** The liver is the principal organ of lipid metabolism and the presence of cirrhosis and hepatocellular carcinoma results in a dramatic shift in the normal metabolism of fatty acids. Among the first HCC metabolomics studies to elucidate this massive deregulation of lipid metabolism in HCC was the Yang *et al*<sup>[28]</sup> work in 2007 which employed high resolution magic angle-spinning <sup>1</sup>H nuclear magnetic resonance

to delineate the metabolomic profile differences in low-grade HCC, high-grade HCC, and non-involved adjacent cirrhosis patient biopsy specimens. The group reported higher levels of several phospholipids in HCC vs cirrhosis, including glycerophosphocholine, phosphatidylcholine, choline and the phosphorylethanolamine. Increases in phosphatidylcholine were further observed in low-grade HCC vs uninvolved cirrhotic tissue, suggesting possible deregulation of glycerophospholipid metabolism at the early phases of HCC development. These phospholipids also exhibited a direct, positive relationship to tumor burden.

In Yang *et al*<sup>[28]</sup>, the elevation of choline, the head



group of many phospholipids that comprise the plasma membrane, not only reflects increased plasma membrane synthesis demand by the growing tumor but is also consistent with the observed elevations in bile (cholic) acid concentration in HCC vs control tissue.

**Lysophospholipids, free fatty acids, and acylcarnitines:** Significant alterations in the expression of a subtype of glycerophospholipids known as lysophosphatidylcholines (LPC) have routinely been observed in HCC metabolomics studies<sup>[29,36-39]</sup>. Still other classes of lipids observed to be significantly altered in HCC vs cirrhosis include free fatty acids (FFA)<sup>[29,45]</sup>, very long chain fatty acids<sup>[29]</sup>, and acylcarnitines<sup>[37,45]</sup>. Down-regulated LPCs and FFA in HCC vs cirrhosis were among the major metabolomic trends reported by Patterson *et al.*<sup>[29]</sup>, with LPC (14:0), LPC (20:3), LPC (22:6) and very long chain fatty acids FFA (24:0) (lignoceric acid) and FFA (24:1) (nervonic acid) all trending lower in HCC vs cirrhosis. The decreases of lignoceric and nervonic acid in HCC vs cirrhosis were especially patent and may reflect peroxisome proliferator-activated receptor-alpha (PPAR- $\alpha$ ) induced enhancement of peroxisomal  $\beta$ -oxidation. Previous reports implicating heightened PPAR- $\alpha$  activity in HCC support this hypothesis<sup>[47,48]</sup>. Decreases in these FFAs may also be related to increased activity of lignoceryl-CoA ligase, the enzyme responsible for very long chain fatty acid catabolism and one acted upon by PPAR- $\alpha$ <sup>[49]</sup>. LPCs are the glycerophospholipid building blocks of cell membranes and elevation of these metabolites may reflect the heightened metabolic needs of growing HCCs. LPCs are also major lipids bound to human albumin<sup>[50]</sup>. Decreased serum albumin is a signature of liver cirrhosis and liver cancer and elevations of systemic LPCs may be attributed to the shortage of appropriate albumin binding sites that results in increased circulating levels of these metabolites.

Further metabolomics work by Xiao *et al.*<sup>[37]</sup> also identified this downregulation of LPCs and LPEs in 40 hepatitis C virus (HCV)-associated HCC patients and 49 cirrhosis controls. Among the novel findings of this investigation included decreased levels of acylcarnitines in HCC vs cirrhosis. Results also showed that as tumor burden worsened, the expression of acylcarnitines and bile acids trended significantly downward. Stage II and III HCC exhibited lower levels of these metabolites in comparison to stage I [staging based on the American Joint Committee on Cancer Tumor Lymph Node Metastatic Disease (TNM) system]. The downregulation of fatty acids, acylcarnitines, and bile acids in HCC vs cirrhosis supports the cancer Warburg effect involving a metabolic shift from tricarboxylic acid (TCA) cycle and mitochondrial  $\beta$ -oxidation to a heightened reliance on glycolysis for energy production. To undergo  $\beta$ -oxidation in the mitochondrial matrix, free fatty acids (fatty acyl-CoA) must link with cytosolic carnitine *via* CPT shuttle system enzymes to form acylcarnitines. Acylcarnitines are capable of penetrating the inner mitochondrial

membrane and once inside the matrix, CPT enzymes liberate fatty acyl-CoA allowing  $\beta$ -oxidation to ensue. Decreased concentration of acylcarnitines in HCC vs cirrhosis suggests impairment of CPT1-mediated formation of these compounds from FFA and carnitine.

**Sphingolipids:** One major lipid expression alteration in HCC reported in HCC metabolomics is a perturbation of sphingosine metabolism, with overexpressed sphingosine-1-phosphate (S1P) and sphingosine reported in HCC vs cirrhosis. The overexpression of LPCs is in agreement with the Patterson study while the upregulation of S1P, a signaling lipid, was a novel finding in HCC metabolomics. S1P has been heavily implicated in promoting the progression of several cancers including HCC<sup>[51,52]</sup> and building the case for this pathway's involvement in HCC development, our HCC metabolomics work identified S1P's precursor sphingosine as one of the most strongly upregulated metabolites in HCC vs cirrhosis<sup>[45]</sup>. Sphingosine is produced *via* acid ceramidase (AC) activity on ceramide. Ceramides are shown to possess apoptotic effects, while sphingosine 1-phosphate is demonstrated as an anti-apoptotic and angiogenic molecule<sup>[53]</sup>. This cell turnover control mechanism is known as the "sphingosine rheostat", and AC is an important modulator of cell death homeostasis. Higher S1P in HCC may also reflect an independent enhancement of sphingosine kinase (SPHK) activity. One study demonstrated the antitumor property of a selective SPHK2 inhibitor in HCC xenografts<sup>[54]</sup>, implicating SPHK as a promoter of HCC progression. Heightened AC activity that results in increased sphingosine may lead to a larger reservoir of S1P *via* sphingosine kinase and may promote the establishment of a microenvironment conducive to HCC initiation.

### **Bile acids**

Bile acids are synthesized in the liver and aid in fatty acid absorption and digestion. Bile acid elevations in HCC have been reported previously<sup>[55]</sup> and may be explained by HCC invasion and obstruction of the bile duct. Bile duct blockage can impede adequate transfer of bile acids to the small intestine thereby impairing sufficient absorption and digestion of fats and leading to a buildup of both bile acids and cholinergic lipids in the hepatic tumor microenvironment. The majority of studies comparing HCC and cirrhosis metabolomes did not uncover a significant differential expression of bile acids, but where a significant trend was seen<sup>[36-38]</sup>, the metabolites collectively trended downward, in contrast to the aforementioned previous reports<sup>[55,56]</sup>. A significant negative correlation between bile acid levels and tumor burden was also observed<sup>[37]</sup>. Similar to the Yang *et al.*'s work, our metabolomics investigation<sup>[45]</sup> identified a significant elevation of choline in HCC vs cirrhosis, and bile acids in our study were strongly elevated in cirrhosis patients vs healthy subjects. The elevation of choline, a building block of bile acids, is consistent with an impairment of bile acid synthesis. Bile acid down-

regulation in HCC may also reflect a metabolic shift away from  $\beta$ -oxidation and the reduced *de novo* bile acid production caused by the obliteration of healthy hepatocytes during chronic liver disease. Diminished bile acids may also reflect constitutive activation of farnesyl X receptor (FXR), a bile acid-activated nuclear receptor that is also activated by a variety of other lipids including eicosanoids<sup>[57]</sup>. FXR silences Cyp7A1-catalyzed production of bile acids and is implicated in promoting progression of HCC by multiple studies<sup>[58,59]</sup>. In the Fujino study of FXR-induced promotion of HCC progression<sup>[58]</sup>, siR-mediated supplementation of FXR enhanced HepG2, Huh7, and HLE HCC cell line progression while FXR knockdown halted this progression.

### **Oxidative stress metabolism**

Among the other pathways found to be significantly associated with HCC in metabolomics investigations are pathways of reactive oxygen species metabolism, notable metabolites of which include the  $\gamma$ -glutamyl peptides. A recent capillary electrophoresis-time of flight mass spectrometry analysis involving sera obtained from HCV-associated HCC patients, cirrhosis patients, hepatitis B virus (HBV) and chronic hepatitis C (HCV) patients, and healthy volunteers showed markedly significant variations in  $\gamma$ -glutamyl expression among these groups<sup>[35]</sup>. No differences in  $\gamma$ -glutamyl peptide expression were observed between HCC and cirrhosis controls, but several significant alterations were witnessed in the HCC vs viral hepatitis, HCC vs normal healthy controls (NHC), cirrhosis vs viral hepatitis, and cirrhosis vs NHC comparisons. In general, HCC  $\gamma$ -glutamyl expression was increased in comparison to healthy controls, while  $\gamma$ -glutamyl peptides were decreased in HCC vs viral hepatitis.  $\gamma$ -glutamylglycine,  $\gamma$ -glutamylalanine,  $\gamma$ -glutamylvaline and  $\gamma$ -glutamylserine,  $\gamma$ -glutamyltaurine,  $\gamma$ -glutamylleucine, and  $\gamma$ -glutamyllysine were all strongly ( $0.0001 < P < 0.001$ ) downregulated in HCC vs viral hepatitis B (HBV) and C infection.  $\gamma$ -glutamyl peptides are precursors to glutathione, the chief antioxidant compound primarily synthesized in the liver. The respective elevation of these intermediates in HCC and cirrhosis patients vs NHC suggests that increased oxidative stress contributing to liver dysfunction calls for heightened production of these precursors to combat this deteriorative process. This is consistent with reports implicating oxidative damage as a key pathway in HCC progression and one that increases patient vulnerability for HCC recurrence<sup>[60,61]</sup>.  $\gamma$ -glutamyl peptides are also liberated in free form by gamma-glutamyl transpeptidase (GGT) mediated breakdown of glutathione. GGT is an enzymatic signature of liver disease and a marker routinely used in the clinic to assess the severity of liver dysfunction. Excess breakdown of glutathione may explain the relative elevation of  $\gamma$ -glutamyl peptides in cirrhosis vs NHC and the impaired oxidative stress neutralization commonly witnessed in HCC.

The Wang *et al.*<sup>[38]</sup> study also found an oxidative stress signature in HCC, showed a striking 677-fold elevation

in canavaninosuccinate (CSA) level in HCC patients vs their cirrhosis counterparts ( $P < 0.01$ ). Subsequent receiver operator characteristic (ROC) analysis revealed that the sensitivity and specificity of AFP and CSA for distinguishing the HCC patients from cirrhosis controls were: AFP<sub>20 ng/mL</sub> 74% and 38%; AFP<sub>200 ng/mL</sub> 52% and 90%; CSA 79.3% and 100%; combined CSA and AFP<sub>20 ng/mL</sub> 96.4% and 100%. CSA is the precursor to fumarate, a key metabolite of the TCA cycle, and elevation of CSA may reflect impairment in the formation of TCA intermediates and promotes the above referenced Warburg theory involving a metabolic shift from oxidative to anaerobic energy production in cancer microenvironments, which are often more hypoxic than healthy tissue<sup>[62]</sup>.

In our work<sup>[45]</sup>, a strong oxidative stress signature in HCC was also observed, with xanthine, 2-hydroxybutyrate and several  $\gamma$ -glutamylpeptides trending significantly higher in HCC vs cirrhosis. Still, the work of Gao *et al.*<sup>[46]</sup> uncovered trends suggestive of an opposite phenomenon: Elevations of glutamic acid, lysine and cysteine in HCC compared with healthy controls were observed and are curious in the context of reactive oxygen species (ROS) given that these amino acids are precursors of glutathione (GSH). Increased levels of these amino acids suggest a possible enhancement of GSH production that establishes a microenvironment conducive for tumor survival<sup>[46]</sup>.

### **Protein metabolites**

The liver is the major organ of protein metabolism and not surprisingly, HCC metabolomics shows that the expression profiles of patients with advanced liver disease exhibit major differences in amino acid expression when compared to metabolic profiles of appropriate diseased or healthy controls. In the Yang *et al.*<sup>[28]</sup> study, significant increases in creatine, glutamine, and glutamate were found in HCC vs cirrhosis, and these trends were juxtaposed by decreases in lactate, alanine, leucine, glutamate, glutamine in HCC vs cirrhosis. The finding of increased amino acids in HCC vs cirrhosis is consistent with numerous studies implicating elevated amino acids and the enzymes responsible for their production in cancer initiation and progression<sup>[63-66]</sup>. In one large scale metabolomics analysis of 60 cancer cell lines, Jain *et al.*<sup>[66]</sup> identified glycine as the most significantly and consistently upregulated metabolite in cancer cells vs healthy lines. Enhanced amino acid production is consistent with the metabolic remodeling hallmark of cancer known as the Warburg effect, which involves a shift from TCA cycle and  $\beta$ -oxidation to a heightened reliance on glycolysis for energy production<sup>[67]</sup>. Amino acids are important glycolytic enzyme activators, and one recent study demonstrated that serine was an activator of pyruvate kinase M2<sup>[68]</sup>, the cancer isoform of glycolytic enzyme pyruvate kinase responsible for conversion of phosphoenolpyruvic acid (PEP) to pyruvate. The group's concomitant observation of elevated lactate in HCC vs cirrhosis is consistent with the Warburg hypothesis.



Gao *et al.*<sup>[46]</sup> propose that their observed elevation of pyruvate, which would seem to counteract the tumor's reliance on the lower-activity PKM2 compared to PKM1, is due to enhanced production of glutamine which is used as an alternative pyruvate precursor. Our study identified a strong amino acid signature associated with HCC with serine, glycine and aspartate being the most significantly upregulated metabolites in HCC vs cirrhosis. These elevations are corroborated by a recent metabolomic analysis of HBV-associated HCC showing a strong upregulation of serine, alanine, glycine, cysteine, aspartic acid, methionine, tyrosine, tryptophan, and phenylalanine in HCC vs healthy controls. Upregulated amino acids in HCC may be explained by the greater protein turnover that transpires in rapidly dividing tumors vs the surrounding non-cancerous tissue along with impaired amino acid utilization in liver.

In addition to very small molecular weight amino acids, metabolomics is also useful for detecting larger protein metabolites weighing near 10 kDa. SELDI TOF-MS metabolomics is a highly versatile methodology for large-scale identification and quantification of these larger metabolites. Wu *et al.*<sup>[31]</sup> tapped this technology to investigate the metabolomic expression profiles of HBV-associated HCC patients, cirrhosis patients, and healthy controls. Their results reveal the upregulation of two proteins, growth related oncogene- $\alpha$  (GRO- $\alpha$ ) and thrombin light chain (TLC), in HCC patients vs cirrhosis controls. To validate these putative markers, serum from an alternative series of HCC, cirrhosis, healthy, and cancer control patients was subjected to Sephadex<sup>TM</sup> Peptide 10/300 GL, Hitrap<sup>TM</sup> CM, and Mono Q 5/50 GL liquid chromatography for protein separation. The separated products were purified using SDS-PAGE and their identities were confirmed using electrospray ionization mass spectrometry. In this alternative series of patients, GRO- $\alpha$  was upregulated four-fold in HCC patients vs cirrhosis controls and its level directly correlated with HCC tumor burden. GRO- $\alpha$  also trended higher in gastric, nasopharyngeal and lung cancers relative to cirrhosis patients, but the magnitude of GRO- $\alpha$  elevation in cancer controls vs cirrhosis was smaller than the elevation witnessed in HCC vs cirrhosis, suggesting that GRO- $\alpha$  may play a more prominent role in HCC progression. Interestingly, TLC was 1.4 times more elevated in HCC patients vs cirrhosis controls but downregulated in the cancer control patients vs cirrhosis, suggesting that TLC is a unique signature of HCC. The combined sensitivity and specificity of GRO- $\alpha$  + TLC + AFP for discriminating HCC patients from cirrhosis and healthy controls was 91.7% and 92.7% respectively. At the 400 ng/mL cutoff for HCC diagnosis, AFP had a sensitivity of 69% and a specificity of 83%.

GRO- $\alpha$  is a chemokine involved in invoking leukocyte cell migration and is associated with pro-inflammatory processes, angiogenesis, and cancer<sup>[69-71]</sup>. Its elevation may be a signature of a viral hepatitis-associated HCC immune deregulation involving heightened monocyte migration and increased inflammation, and a subsequent

increased likelihood for successful host evasion of tumor prophylactic mechanisms<sup>[72]</sup>. TLC is a protein cleavage fragment that is generated from matrix metalloproteinase-associated (MMP) peptide cleavage<sup>[31]</sup>. Its upregulation may be explained by simultaneous E-cadherin loss and MMP activation by Twist1 which has been shown to promote HCC expansion<sup>[73]</sup>. Its utility as a cancer biomarker was also shown in a SELDI TOF-MS study of gastric cancer by Ebert *et al.*<sup>[74]</sup>, where TLC accurately distinguished gastric cancer patients from patients without cancer with a sensitivity of 89.9% and a specificity of 90%.

The destruction of cholinergic receptors in amyloid plaques and neurofibrillary tangles of the brain has also been linked to encephalopathy, a common neurological disorder witnessed in HCC patients<sup>[75]</sup>. The molecular mechanisms linking hepatic encephalopathy (HE) and HCC are not fully understood, but the parallel elevations of choline and glutamine in HCC patients vs cirrhosis controls may partially be associated with a cataclysmic loss in cholinergic receptor concentration and the initial stages of HE onset, respectively<sup>[76]</sup>. A possible neurological metabolic signature was one of the most strongly correlated trends observed in HCC patients vs cirrhosis and vs healthy controls in our study, with 2-pyrrolidinone, a  $\gamma$ -aminobutyric acid (GABA) metabolite, was strongly elevated in HCC when compared to levels in both the cirrhosis controls and the healthy volunteers. GABA is a major inhibitory chemical messenger in the brain, and the sharp elevation of its metabolic byproducts in HCC may reflect heightened production of GABA and may coincide with the neurodegenerative hallmarks of advanced liver disease.

Two previously unidentified fibrinogen cleavage peptides, denoted by amino acid sequence as DSGEGD FXAEGGGVR and ADSGEGDFXAEGGGVR, were observed to be significantly overexpressed in HCC in our study. While they trended higher in HCC vs cirrhosis, their elevation in cirrhosis vs healthy controls was more patent and these metabolites exhibited a better diagnostic utility for cirrhosis. Taken together, the aberrations in protein and amino acid expression in HCC revealed by metabolomics may be harnessed toward the development of clinically fruitful HCC diagnostics.

### Markers of cirrhosis

A number of HCC metabolomics studies report significant alterations between cirrhosis patients and healthy volunteers<sup>[30,32,33,35,38,39,41,42,45,46]</sup> and within these findings are opportunities for development of biomarkers for cirrhosis. Currently, the best cirrhosis diagnostic is liver biopsy, a procedure that by virtue of its invasive nature is not a gold standard diagnostic approach<sup>[77]</sup>. Identification of accurate cirrhosis diagnostic biomarkers can streamline development of a less invasive diagnostic that minimizes patient discomfort and the expenses associated with biopsy while simultaneously enabling early detection of cirrhosis in both industrialized nations and in resource poor areas of the globe. Among the putative markers

of cirrhosis identified in these works include the purine metabolites hypoxanthine<sup>[41,45]</sup> and inosine<sup>[41]</sup>, both significantly downregulated five and six-fold respectively in cirrhosis vs NHC.  $\gamma$ -glutamylalanine,  $\gamma$ -glutamylvaline,  $\gamma$ -glutamylglutamine,  $\gamma$ -glutamylphenylalanine and  $\gamma$ -glutamylcitrulline were also significantly elevated in cirrhosis vs NHCs<sup>[41,45]</sup>. Increases in the  $\gamma$ -glutamyl peptides in cirrhosis vs NHC and HCC vs NHC indicates heightened production of glutathione to combat the oxidative damage process commonly implicated as a promoter of tumor initiation and progression.  $\gamma$ -glutamyl peptides are also liberated in free form by GGT mediated breakdown of glutathione. The elevation of  $\gamma$ -glutamyl peptides in cirrhosis vs NHC may therefore conversely be explained by excessive breakdown of glutathione resulting in impaired oxidative stress neutralization that is commonly witnessed in HCC.

Other promising markers of cirrhosis include bile acids<sup>[45]</sup> and dicarboxylic acids<sup>[45,46]</sup>, with these metabolite classes exhibiting the strongest and most significant fold-differences among all markers significantly altered between cirrhotics and healthy controls in the references metabolomic studies.

### **Etiological metabolomic differences**

Because HCC is linked to a variety of diverse etiologies including viral hepatitis, alcoholic cirrhosis, non-alcoholic fatty liver disease, steatohepatitis, and aflatoxin B1, metabolomics is useful for revealing the variation in metabolism these etiologies cause. The majority of studies have focused on characterizing the metabolomic signatures of single HCC etiologies, or have not accounted for etiology. Studies to date have not queried the metabolomic expression pattern differences between the various etiologies of HCC and as the metabolic underpinnings of each etiology are better characterized, HCC etiology metabolome comparison studies will prove vital in establishing the utility for metabolomics as an accurate diagnostic modality of different HCC subtypes. Only one study, by Zhou *et al.*<sup>[39]</sup>, compared the serum metabolomic expression patterns of HCV cirrhosis-associated HCC and HBV cirrhosis-HCC. The group also looked at whether metabolomic alterations in HCV-HCC and HBV-HCC were significantly different from cirrhosis and healthy control metabolomes. The study revealed a greater magnitude decrease of LPC expression in HBV-infected HCC patients vs cirrhosis controls than the magnitude decrease of LPCs HCV-HCC vs cirrhosis. The analysis also showed significant elevation of bile acids, heme pigmentation compounds bilirubin and biliverdin, upregulation of acylcarnitines and downregulation of glycerophospholipids in cirrhosis patients vs healthy controls, suggesting that these metabolites were signatures of the onset of cirrhosis. Metabolomic profile comparison between HBV-cirrhosis patients vs patients with HBV-only revealed a similar global downregulation of LPCs in the cirrhosis cohort vs viral hepatitis controls. The findings of this study suggest a progressive downregulation of LPCs during the course of progression

from viral hepatitis to cirrhosis and a bottomed out expression occurring with HCC. The downregulation of LPC in HCC may be explained by their well-known anti-tumor roles that include induction of apoptosis, anti-invasive effects, and a direct effect on tumor sensitization to treatment. This trend of diminished LPCs may reflect first the substantial cell death that occurs during cirrhosis resulting in diminished LPC levels, followed by a subsequent massive turnover of residual LPCs by the growing HCC that further exhausts the LPC reservoir. The magnitude of LPC decrease in HBV-HCC vs cirrhosis was greater than HCV-HCC vs cirrhosis, suggesting that HBV exerts a more prominent influence on LPC metabolism than HCV.

### **Integrated “omics” approaches**

As the above studies demonstrate, metabolomics is a powerful strategy for identifying a large panel of metabolites that exhibit promise in accurately diagnosing HCC. What is unclear from these works, however, are the genomic and proteomic synergies that culminate in these metabolic manifestations. Integrating two or more “omics” approaches can unveil the complex genomic-proteomic-metabolomic network galvanizing cancer development. Two recent studies tapped the power of such an approach, coupling metabolomics with transcriptomics to identify the genetic underpinnings of metabolomic disruptions. The first study by Budhu *et al.*<sup>[43]</sup> investigated the network of metabolic pathways and corresponding genes involved in HCC aggressivity. Patient demographic and clinical characteristics, including body mass index, were extensively characterized for 356 HCC cases, which were divided into a training set ( $n = 30$ ), a testing set ( $n = 217$ ), and a validation cohort ( $n = 139$ ). The training cohort consisted of 15 epithelial cell adhesion molecule-positive/AFP-positive (EpCAM+AFP+) HpSC-HCC patients, representing aggressive stem-like HCC, and 15 less aggressive (EpCAM-AFP-) mature hepatocyte MH-HCC patients. Analysis of tumor specimens and uninvolved healthy tissue by principal component analysis revealed clear demarcation between tumor and non-tumor metabolomes. Non-targeted metabolic profiling of tumor and non-tumor specimens identified 48 markers that were significantly altered between HpSC-HCC patients and patients with MH-HCC. This metabolite panel resolved aggressive HCC from the less subtype at a sensitivity of 72% and a specificity of 83% ( $P < 0.05$ ). The group further demonstrated that within the subset of 48 metabolites associated with HCC aggressivity, 28 were significantly associated with overall survival. Subsequent microarray gene expression profiling of the paired tumor specimens identified 169 genes that could significantly distinguish the two HCC subtypes. The group then gauged the correlation between this genetic signature and the panel of 28 metabolites that were associated with both HCC aggressivity and overall survival and found that each of the 28 metabolites were associated with at least one of the 169 genes identified by microarray analysis. To determine the principal metabolite-gene

pairs potentially influencing HCC aggressivity, the group performed correlation analysis with randomization and found 15 metabolites and 121 genes most highly associated with the tumor and stem-like HCC genes. Validation of the genetic signature in an independent testing cohort ( $n = 217$ ) revealed a panel of 273 genes that distinguished HpSC-HCC from MH-HCC with a sensitivity of 72% and a specificity of 91% ( $P < 0.01$ ), and this genetic signature was also strongly ( $P < 0.0001$ ) associated with overall survival. This gene set was also a significant independent predictor of overall survival, progression free survival and recurrence, highlighting its utility as a prognostic panel of aggressive HCC. Many of the genes were associated with fatty acid metabolism and the phosphatidylinositol 3-kinase signaling pathway. Elevated palmitoleate expression in HpSC-HCC tumor vs paired non-tumor specimens reflected overexpression or enhanced activity of stearoyl-CoA dehydrogenase (SCD), an important cell turnover regulatory pathway. Significant downregulation of arachidonate and linoleate, the parent molecules of the eicosanoid signaling cascade, suggested overconsumption of these metabolites from hyperactivity of cyclooxygenase, lipoxygenase, and/or cytochrome P450c in aggressive HCC. SCD, which converts saturated palmitic acid to palmitoleic acid, was among the gene set. To validate the possible role of SCD in cancer aggressivity, *in vitro* and *in vivo* analysis was done and showed that selective SCD inhibitor CGX0168 abrogated Huh7 cell migration and invasion. Supplementation of Huh7 with palmitoleate, the end product of SCD activity, enhanced cell migration and invasion. Xenografted tumors in nude mice showed that SCD-siRNA inhibited tumor migration and colony formation and increased apoptosis, likely owing to the accumulation of pro-apoptotic palmitate. Taken together, this approach showed a strong lipid signature associated with HCC aggressivity. Elevated palmitoleate reflected upregulated SCD, a key regulator of the ratio between saturated and unsaturated fatty acids. A tilt in the delicate balance between saturated and unsaturated fatty acids toward unsaturated has been implicated in cancer aggressiveness.

Remarkably, the integrated metabolomics-transcriptomics study by Beyoğlu *et al.*<sup>[44]</sup> also revealed a consistent role for palmitate, linoleate, and the PI3K pathway in HCC. Microarray analysis found that 11 genes involved in fatty acid were associated with HBV-positive HCC cases. More broadly, the comparison of tumor vs paired non-tumor tissues identified a metabolic shift toward glycolysis reflective of the Warburg effect implicated in other HCC metabolomics works. Levels of glucose, glycerol 3-phosphate, glycerol 2-phosphate, malate, alanine, and myo-inositol were all significantly decreased in tumor vs non-tumor specimens, indicating both impaired mitochondrial respiration and enhanced glycolysis. Myo-inositol is the metabolic precursor of the messenger molecule inositol triphosphate (PI3) required by PI3K-AKT-mTOR, and its downregulation in tumor vs non-tumor specimens is consistent with the hyperactivity of this pathway implicated in numerous cancers. Further-

more, the group showed that 1-stearoylglycerol and 1-palmitoylglycerol were decreased in tumor vs non-tumor. 1-acylglycerols are synthesized by phospholipase activity on LPCs and the routinely reported decrease in LPCs in HCC vs controls may explain the reduced 1-acylglycerols in this study. Through integration of metabolomics and transcriptomics, these groups together show a possible role for SCD in HCC, implicate the PI3K pathway as important to HCC progression, and show a strong lipid signature associated with HCC, in agreement with other HCC metabolomics works. More broadly, these works demonstrate that the achievement of consistent trends in independent, integrated “omics” studies can be achieved and lay important groundwork for future studies of this nature.

### **Sensitivity and specificity of metabolomics in HCC diagnosis**

To evaluate the utility for metabolomic profiles to distinguish between patients with cirrhosis and patients with HCC, these studies primarily employed principal component analysis, orthogonal projection to latent structures, supervised projection to latent structures discriminant analysis, the random forest machine learning algorithm, or ROC curve class prediction analyses. Reported sensitivity/specificity/area under the curve values reflecting the accuracy of metabolomics in distinguishing HCC from cirrhosis are shown in Table 3. In general, metabolomics was a highly accurate diagnostic method and clear demarcation between healthy controls and/or cirrhosis controls vs HCC patients were realized. Where metabolomics profile class prediction was compared with AFP, the metabolomics approach showed greater class prediction power. In general, the focus of these works was the potential standardization of metabolomics as a high-throughput clinical diagnostic platform-big-data biomarkers rather than single metabolite alterations—which may explain the lack of emphasis on the sensitivity and specificity of individual metabolites for diagnosing HCC. Just two studies verified the metabolite expression patterns *in vitro*, and with the exception of the *in vivo* validation performed by Budhu *et al.*<sup>[43]</sup>, no studies used *in vivo* models to validate their metabolomics data. It is therefore recommended that future validation of HCC metabolomics data include preliminary information on metabolite concentrations in HCC cases vs cirrhosis controls as measured *in vitro* or *in vivo* to reveal their utility as potential biomarkers that can be employed in a rapid *in vitro* diagnostic.

## **DISCUSSION**

### **Summary of major findings**

The results of these studies show that aberrations in bile acid, LPC, acylcarnitine, ROS, and protein metabolism may be signatures of HCCs emerging in the setting of cirrhosis. Bile acids trended lower in HCC vs cirrhosis controls in all studies reporting a significant difference in expression of these metabolites<sup>[36-38]</sup>. In general, LPCs were shown

**Table 3** Utility of significantly altered ( $P < 0.05$ ) metabolites in accurately predicting hepatocellular carcinoma (hepatocellular carcinoma cases *vs* patients with cirrhosis)

Ref.	Platform	Comparison	Class prediction methodology	Classification accuracy or sensitivity/specificity	AFP sensitivity /specificity
Patterson <i>et al</i> <sup>[29]</sup>	UPLC/ESI-QTOF-MS	HCC ( $n = 20$ ) <i>vs</i> cirrhosis ( $n = 7$ )	Random forest	96.3	-
Chen <i>et al</i> <sup>[30]</sup>	Integrated GC/QTOF-MS + UPLC/QTOF-MS	HCC ( $n = 82$ ) <i>vs</i> healthy ( $n = 71$ )	OPLS-DA	100.0	-
Wu <i>et al</i> <sup>[31]</sup>	SELDI-TOF MS	HCC ( $n = 48$ ) <i>vs</i> cirrhosis ( $n = 54$ ) or healthy ( $n = 42$ )	GRO- $\alpha$ + thrombin light chain PS20 Protein immunoassay	89.6/89.6	69/83
Cao <i>et al</i> <sup>[32]</sup>	UPLC/QTOF-MS	HCC ( $n = 23$ ) <i>vs</i> cirrhosis ( $n = 22$ )	PLS-DA	67.0	-
Gao <i>et al</i> <sup>[33]</sup>	NMR	HCC ( $n = 39$ ) <i>vs</i> cirrhosis ( $n = 36$ )	PLS-DA	45.7	-
Wu <i>et al</i> <sup>[34]</sup>	GC/MS	HCC ( $n = 20$ ) <i>vs</i> healthy ( $n = 20$ )	PCA with ROC curve analysis	AUC=88.3; AUCAFP = 92.5 when combined with AFP	-
Soga <i>et al</i> <sup>[35]</sup>	LC/MS-MS	HCC ( $n = 32$ ) <i>vs</i> HCV-only ( $n = 35$ ) or cirrhosis ( $n = 18$ )	Multiple logistic regression; ROC curve analysis	88.1	0.760
Wang <i>et al</i> <sup>[38]</sup>	UPLC-MS	HCC (59) <i>vs</i> cirrhosis (20) or NHC (20)	PLS-DA, ROC curve analysis	CSA 79.3/100 CSA + AFP20 96.4/100 UPLC-MS 100/100	AFP20 74/38 AFP200 52/90
Zhou <i>et al</i> <sup>[39]</sup>	UPLC-QTOF-MS	HCC ( $n = 69$ ) <i>vs</i> cirrhosis ( $n = 28$ )	PLS-DA, ROC curve analysis	AEA 88.0 PEA 82.0 AEA + PEA 88.0	-
Nahon <i>et al</i> <sup>[40]</sup>	NMR	Small HCC ( $n = 28$ ) <i>vs</i> cirrhosis ( $n = 93$ ); Large HCC ( $n = 33$ ) <i>vs</i> cirrhosis ( $n = 93$ )	OPLS	Small HCC: 61.0/100.0 Large HCC: 100.0/100.0	-
Yin <i>et al</i> <sup>[41]</sup>	RPLC/QTOF-MS; HILIC/QTOF-MS	HCC ( $n = 25$ ) <i>vs</i> cirrhosis ( $n = 24$ ) or healthy ( $n = 25$ )	OPLS	RPLC: 61.8 HILIC: 57.0 RPLC + HILIC = 63.6	-
Li <i>et al</i> <sup>[42]</sup>	UPLC/QTOF-MS	HCC ( $n = 8$ ) <i>vs</i> cirrhosis ( $n = 6$ ) or healthy ( $n = 6$ ) (murine samples)	OPLS-DA	88.2	-
Budhu <i>et al</i> <sup>[43]</sup>	Training set1: GC/MS + UPLC/MS-MS; Testing set2: Affymetrix GeneChip	Training set: Stem-like aggressive HpSC-HCC ( $n = 15$ ) <i>vs</i> Mature hepatocyte less aggressive MH-HCC ( $n = 15$ ); Testing set: HpSC-HCC and MH-HCC ( $n = 217$ )	Multivariate analysis	172.0/83.0, AUC = 0.830 272.0/91.0, AUC = 0.860	-
Fitian <i>et al</i> <sup>[45]</sup>	UPLC/MS-MS + GC/MS	HCC ( $n = 30$ ) <i>vs</i> HCV-cirrhosis ( $n = 27$ )	Random forest  ROC analysis	72% 12-HETE 73.3/69.2 15-HETE 83.3/59.3 Aspartate 100/51.9 Glycine 83.3/63.0 Serine 73.3/85.2 Phenylalanine 73.3/81.5 Homoserine 70.0/85.2 Sphingosine 58.3/86.7 Xanthine 63.3/88.9 2-Hydroxybutyrate 76.7/77.8	AFP20 63.3/83.6
Gao <i>et al</i> <sup>[46]</sup>	GC-TOF/MS	HCC ( $n = 39$ ) <i>vs</i> HBV-cirrhosis ( $n = 52$ )	Random forest (validation set)  ROC analysis (validation set)  Bayes discriminant function model (validation set)	96.8% in HCC <i>vs</i> HBV-cirrhosis 100% in HBV-cirrhosis <i>vs</i> HBV 100% in HBV <i>vs</i> NHC 100/95.2 HBV <i>vs</i> NC 83.3/100 HBV-cirrhosis <i>vs</i> HBV 76.9/83.3 HCC <i>vs</i> HBV-cirrhosis 76.9% HCC 100% HBV-cirrhosis 94.1% HBV 100% NHC	-

Classification accuracy describes the capacity of the metabolomic classification technique to accurately predict the group of each study subject. UPLC: Ultrahigh-performance liquid chromatography; AEA: Anandamide; OPLS: Orthogonal projection to latent structure; PCA: Principal component analysis; PEA: Palmitylethanolamide; PLS-DA: Partial least squares-discriminant analysis; MS: Mass spectrometry; TOF: Time-of-flight; GC: Gas chromatography; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; GRO- $\alpha$ : Growth related oncogene-alpha; ROC: Receiver operator characteristic; NHC: Normal healthy controls; AFP20: AFP performance at the cutoff of 20 ng/mL; AFP200: AFP performance at the cutoff of 200 ng/mL.



to be downregulated in HCC vs cirrhosis<sup>[37-39,45]</sup>. But the works by Resson *et al.*<sup>[36]</sup> and Wang *et al.*<sup>[38]</sup> found that LPCs and LPEs were consistently elevated in the HCC vs cirrhosis comparison. Acylcarnitines, which function as liaisons of fatty acid  $\beta$ -oxidation by enabling fatty acid importation to the mitochondrial matrix, were significantly downregulated in HCC vs cirrhosis. Amino acids also trended higher in HCC in all studies reporting significant expression differences between HCC and cirrhosis. The hemoglobin metabolite bilirubin was also consistently elevated in HCC vs cirrhosis<sup>[29,39,41,45]</sup>, consistent with the clinical utility this metabolite possesses for diagnosing advanced liver disease. Biliverdin, another heme catabolite, was significantly upregulated in HCC vs cirrhosis in two studies<sup>[29,45]</sup> and a metabolic derivative of biliverdin downregulated in a third<sup>[37]</sup>.

The elevation of LPCs in HCC vs cirrhosis seen by Resson *et al.*<sup>[36]</sup> is consistent with increased demand of glycerophospholipids by the growing tumor. LPCs comprise only 3% of the total phospholipids in plasma membranes, however, and a better explanation for their elevation may be that LPCs are major lipids bound to albumin. The loss of albumin commonly witnessed in chronic liver disease and HCC may mean a shortage of docking sites for LPCs, resulting in increased systemic levels of these glycerophospholipids. Diminished bile acids in HCC vs cirrhosis reinforces the Warburg effect commonly witnessed in cancer studies involving a shift in energy production away from oxidative processes like  $\beta$ -oxidation and the TCA cycle toward anaerobic glycolysis, which is more suitable to the hypoxic or anoxic tumor microenvironment. The loss of bile acids in HCC significantly impairs fatty acid absorption and digestion and is consistent with the general trend of increased circulating lipids in HCC vs cirrhosis and cirrhosis vs appropriate controls. The elevation of bile acids has historically served as a clinical indicator of chronic liver disease and specifically cirrhosis and consistent with this function, our metabolomics work showed a global upregulation of bile acids in cirrhosis vs healthy volunteers<sup>[45]</sup>. Decreased acylcarnitines further reflects diminished reliance on  $\beta$ -oxidation, and it may also signify impairment of the mitochondrial carnitine palmitoyltransferase shuttle system.

### **Metabolomic heterogeneity attributed to differences in study design**

While perturbations in phospholipid, bile acid, hemoglobin, acylcarnitine and amino acid metabolism were routinely encountered in these studies, the referenced works did not achieve uniform conclusions regarding the directional shifts of the metabolites' expression in HCC vs cirrhosis, with examples including contradictory patterns of LPC<sup>[36,39]</sup> and amino acid<sup>[28,46]</sup> expression in HCC vs cirrhosis. The heterogeneity among these studies' metabolomes is likely due to differences in study design. Although some studies matched patients by age and gender, the majority of the referenced HCC metabolomics

studies did not extensively characterize HCC and cirrhosis patients by demographic and clinical characteristic parameters. Only four studies reported MELD scores for HCC and cirrhosis patients<sup>[36,37,40,45]</sup>, and just three studies indicated the Child-Pugh status of their HCC or cirrhosis cohorts<sup>[29,40,45]</sup>. This lack of information on whether liver function of the study participants is compensated or decompensated complicates interpretation of metabolomic data. Body mass index (BMI) also went unreported for HCC, cirrhosis, and healthy study subjects in all but two studies<sup>[43,45]</sup> referenced in this review. BMI can have a significant influence on the relative metabolite expression differences between patients, particularly with regard to adiposity. Because these studies did not control for BMI, is likely that the patients recruited for these metabolomics studies had wide-ranging BMIs that may further explain the noticeably different trends of LPC, FFA, and acylcarnitine expression in HCC vs controls. To limit the influence of potential cofounders such as comorbidity, BMI, age or gender, and etiology on HCC metabolomes, patient clinical characteristics should be controlled for more conscientiously in future HCC metabolomics studies.

There was also marked variation in how each group diagnosed and staged their HCC patients. Three studies<sup>[29,43,45]</sup> staged according to the Barcelona Clinic for Liver Cancer staging criteria. The TNM Classification of Malignant Tumors (TNM) was used to diagnose HCC in five studies<sup>[28,30,37,38,43]</sup>, imaging in two, histopathology in one<sup>[36]</sup>, and six studies made no mention of their HCC diagnosis method<sup>[31,33,35,39,41,42]</sup>. Differences in HCC diagnosis and staging among these studies likely contributed to the discordant global metabolomic alterations in HCC vs cirrhosis among these studies and further confounded the interpretation of these metabolomic trends.

### **Lack of emphasis on HCC vs cirrhosis comparison**

The critical metabolomic comparison between HCC patients and cirrhosis controls was reported in just ten out of the twenty studies referenced in this review<sup>[28,29,31,35-39,45,46]</sup>, with half of the cited works instead focusing on the metabolomic profile differences between HCC patients and NHC subjects. While this comparison sheds light on altered pathways during hepatocarcinogenesis, it may not be as applicable from a clinical standpoint as the trends elucidated through a comparison of HCC vs cirrhosis. This owes to the fact that the majority of primary liver cancer cases occur in patients with a preexisting condition of cirrhosis. Therefore, the metabolomic comparison between HCC and cirrhosis is more clinically informative and potentially translational than the comparison of HCC vs NHC. HCC is a complex heterogeneous disease and HCC patients often present with multiple comorbidities. It is therefore likely that marked metabolomic differences will be observed between HCC patients and healthy subjects. Differences between HCC patients and cirrhosis controls are subtler than HCC vs NHC<sup>[45,46]</sup> and it would be expected that conspicuous metabolomic differences



exist between HCC/NHC and cirrhosis/NHC. Given that HCC arose in the background of cirrhosis in all reported HCC metabolomics studies, it is impossible to determine whether the alterations witnessed in HCC vs NHC are related to HCC or cirrhosis, further reinforcing the need for future metabolomic comparisons between HCC and cirrhosis.

## CONCLUSION

Bile acids, acylcarnitines, amino acids, free fatty acids, LPCs, and heme pigmentation molecules exhibited utility in stratifying HCC patients from patients with cirrhosis. Canavaninosuccinate, which showed a striking 680-fold elevation in HCC patients vs cirrhosis controls and outperformed AFP in sensitivity and specificity. Sphingosine 1-phosphate (↑), sphingosine (↑), GRO- $\alpha$  (↑), and thrombin light chain (↑) were other putative HCC biomarkers that had superior predictive utility for HCC than AFP. Bile acids, fibrinogen cleavage byproducts, dicarboxylic fatty acids, and ROS-related  $\gamma$ -glutamyl peptides exhibited strong association with cirrhosis and the further development of these metabolites as diagnostic markers of cirrhosis may be valuable. In addition to individual metabolites, global patient metabolomes exhibited superior sensitivity for diagnosis of HCC vs AFP where a comparison was made<sup>[31,35,38,45]</sup>.

Although these data are preliminary in nature, they reflect the early promise that metabolomics platforms hold in potential clinical implementation for disease diagnosis. Future metabolomics studies with larger, better demographically and clinically characterized patient cohorts may resolve the heterogeneous metabolomic expression patterns in HCC vs cirrhosis.

Given that > 90% of HCCs emerge in the setting of cirrhosis, future HCC metabolomics studies will be most impactful and clinically relevant if they compare the expression patterns of HCC patients vs cirrhosis controls. Moreover, patients with HCC and cirrhosis should be matched by etiology (viral vs non-viral), liver function, and BMI to limit the influence of data confounders. Furthermore, it is paramount the appropriate metabolomic profile comparisons are made, namely HCC vs cirrhosis, cirrhosis vs etiology of cirrhosis (viral or non-viral), and cirrhosis vs NHC. In a recent study, the entire stepwise hepatocarcinogenic process from NHC to HBV, through cirrhosis, and culminating in HCC was analyzed. Appropriately, Gao *et al.*<sup>[46]</sup> focused their expression profile comparisons on HCC vs cirrhosis, and cirrhosis vs HBV/NHC. This approach enables investigators to make adequate conclusions about the interval at which putative biomarkers become relevant in the hepatocarcinogenic process and demonstrates the relevance of the dataset comparisons. Moreover, investigation of the pathways involved in the progression from the initial liver insult to cirrhosis remains a largely untapped realm of biomarker discovery within metabolomics, and may streamline the identification of potential cirrhosis diagnostic markers. More broadly, metabolomics is well-suited for clarifying

the entirety of the metabolic remodeling that occurs throughout hepatocarcinogenesis, and can hence streamline biomarker discovery efforts at each pathological interval.

The findings of these works demonstrate the powerful resource that is metabolomics for identifying potential novel diagnostic biomarkers of HCC. The translational optimality of metabolomics is underscored by its capability to simultaneously process high volumes of patient specimens and interpret metabolic expression profiles through robust, validated and automated software. Still greater, metabolomics holds promise as a novel disease screening and diagnostic modality that, through characterization of a patient's global metabolic profile, can in a more sophisticated and comprehensive fashion accurately predict the presence of disease.

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