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Serum Amino Acid Profiles Predict the Development of Hepatocellular Carcinoma in Patients with Chronic HBV Infection

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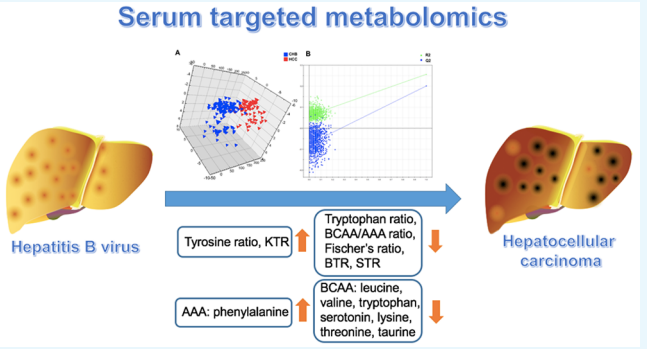
ABSTRACT: ***Background:*** The study aimed to find out the alterations in serum amino acid (AA) profiles and to detect their relationship with carcinoma formation. ***Methods:*** Targeted metabolomics based on ultraperformance liquid chromatography triple quadrupole mass spectrometry to quantitatively analyze serum AA levels in 136 hepatitis B (CHB) patients and 93 hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) patients. ***Results:*** It was shown that decreased serum levels of leucine, lysine, threonine, tryptophan, valine, serotonin, and taurine were observed in more HCC patients than CHB patients, but the serum phenylalanine level was increased. Serum valine and serotonin were lower in Class C than Class A and Class B in HCC patients. Accompanied with the higher score of Model for End-Stage Liver Disease, serum phenylalanine was increased not only in CHB patients but also in HCC patients. The serum level of phenylalanine increased in the decompensated stage more than in the compensated stage, while serum leucine and serotonin significantly decreased. Serum serotonin still had significant differences between CHB and HCC both in the HBV desoxyribonucleic acid (HBV-DNA) negative group and in the HBV-DNA positive group. Furthermore, it was shown that the tryptophan ratio, branched-chain amino acids (BCAA)/aromatic amino acids ratio, BCAAs/tyrosine ratio, Fischer's ratio, and serotonin-to-tryptophan ratio significantly decreased, while the tyrosine ratio and the kynurenine-to-tryptophan ratio increased in HCC patients more than those in CHB. ***Conclusions:*** A distinct metabolite signature of some specific serum amino acids was found between CHB and HCC patients, which may help predict the development of HCC at an early stage.



BACKGROUND

Nowadays, there are ∼250 million people who are infected with hepatitis B virus (HBV) all over the world,[1] and it can progress to cirrhosis and hepatocellular carcinoma (HCC). HCC is the third major cause of cancer death around the world. Major causes of HCC include viral infections, toxic, metabolic, and immune factors.[2] HCC shows a poor prognosis and is short of effective therapeutic procedures.[3,4] HCC has become one of the most prevalent malignancies resistant to current chemotherapies or radiotherapies.

However, the potential mechanism of HBV-induced HCC remains unclear and requires further investigation. Thus, it is urgent to explore the metabolite changes related to HCC diagnosis and screening, in order to identify significant biomarkers associated with disease progression and provide potential new targets for effective drug discovery.

Metabolomics provides global metabolic information to analyze physiological and pathological states, considering both the intrinsic characters of the bodies and the outside effects.[5] In recent years, metabolomics has been widely applied in the study of liver disease. McPhail et al.[6] concluded that plasma lysophosphatidylcholines (lysoPCs) and amino acid (AA) dysregulation contributed to the increased mortality and severity in decompensated cirrhosis. Andrea et al.[7] found that serum glucose, lactate, lipids, alanine, 1-methylhistidine, glutamine, valine, and lysine levels were significantly changed between early (n = 28) and advanced (n = 36) HCC patients. Our former study showed that serum long-chain lysoPC at C18:2, C20:3, C20:4 progressively decreased from CHB to cirrhosis to HCC.[8]

However, less is known of the changes of AAs during infection in HBV-associated hepatitis and carcinoma. Therefore, it is remarkably important to further identify novel and specific biomarkers for the diagnosis of HCC as well as novel potential targets.[9] In the present study, we performed targeted

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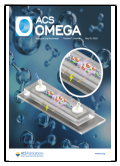

Table 1. Demographic Information and Clinical Characteristics of Patients[a]

factors	CHB	HBV-associated HCC	*P*-value
N	136	93	
age (ys)	41(32−52)	56(47−62) ***	0.000
gender (F/M)	51/85	14/79 ***	0.000
BMI (kg/m^2)	23.66(21.96−24.77)	22.31(19.61−24.17) ***	0.000
ALT (U/L)	55.5(31.75−109.25)	40(25−65)	0.060
AST (U/L)	44(27−88.25)	48(32−104)	0.252
TBIL (mM)	14.9(11.47−21.47)	28.5(14.4−43.2) **	0.003
DBIL (mM)	5.4(3.5−8.35)	9(3.9−18.1) *	0.013
IBIL (mM)	9.95(7.3−13.43)	16.3(9.7−25.3) **	0.001
ALP (U/L)	66.28(4.5−95.5)	120(83−208) ***	0.000
GGT (U/L)	54.5(27.75−111.25)	110(47−234) ***	0.000
TP (g/L)	73.45(69.68−76.82)	68.5(64.1−73.5) **	0.002
ALB (g/L)	42.4(39.32−45.32)	36.1(31.3−40.8) ***	0.000
PALB (g/L)	0.17(0.12−0.23)	0.1(0.06−0.16) ***	0.000
GLB (g/L)	30.3(27.58−33.82)	32.1(27.7−35.6)	0.054
LDH (U/L)	200.75 (160−364.25)	198(170−249) *	0.010
TBA (mM)	17.95(5.75−24.78)	37.6(12.4−77.7) ***	0.000
CHE (U/L)	6521.98 (4872.5−8114.5)	4061(2549−5765) ***	0.000
CREA (mM)	58.92(47.65−70.35)	68.1(54.8−80.6) ***	0.001
BUN (mM)	4.16(3.43−4.98)	4.91(4.05−6.37) ***	0.000
UA (mM)	281.12 (221.5−333.5)	307(249−376) *	0.026
TC (mM)	4.08(3.44−4.51)	3.7(3.2−4.3) **	0.006
TG (mM)	1.2(0.95−1.44)	1(0.7−1.2) ***	0.000
HDL-C (mM)	1.19(0.95−1.41)	1.08(0.83−1.4)	0.622
LDL-C (mM)	2.2(1.7−2.62)	2.21(1.76−2.58)	0.825
VLDL (mM)	0.69(0.27−0.9)	0.25(0.15−0.4) **	0.005
ApoA1 (g/L)	1.36(1.2−1.55)	1.06(0.85−1.28) ***	0.000
ApoB (g/L)	0.76(0.65−0.9)	0.75(0.6−0.83)	0.480
FFA (mM)	0.3(0.15−0.49)	0.58(0.38−0.78) ***	0.000
GLU (mM)	5.2(4.8−5.63)	5.29(4.89−5.87)	0.068
PT (Sec)	12.5(12.07−13.2)	14(13.1−15.2) ***	0.000
PTR (%)	1.09(1.04−1.14)	1.22(1.13−1.3) ***	0.000
APTT (Sec)	29.7(27.8−31.42)	32.79(29.7−34) **	0.004
TT (Sec)	19.03(18.3−19.7)	18.73(17.5−19.3)	0.423
FIB (g/L)	2.1(1.8−2.3)	2.75(2−3.2) ***	0.000
INR (%)	1.09(1.04−1.14)	1.22(1.12−1.3) ***	0.000
AFP (ng/mL)	5.72(3.33−14.27)	44.45(6.88−1181.47) *	0.011
CEA (ng/mL)	2.1(1.48−2.8)	2.8(1.8−3.33) **	0.006
HBV DNA (Log IU/mL)	4.64(0−7.25)	0(0−3.24) ***	0.000
RBC (10^{12}/L)	4.44(4.15−4.74)	3.9(3.4−4.45) ***	0.000
WBC (10^9/L)	5.1(4.2−5.6)	4.44(3.18−6.7)	0.794
HCT (%)	41.55(39.4−44.35)	36.45(31.7−41.3) ***	0.000
HGB (g/L)	139.7(132−152)	125.43(109−142) ***	0.000
MCH (pg)	31.53(30.7−33)	32.4(31.3−33.9) *	0.028
MCHC (g/L)	338(334.75−342)	340.63(336−345)	0.126
MPV (fL)	9.75(8.88−10.53)	9.6(8.6−11.5)	0.782
PLT (10^9/L)	160.3(123−189.5)	104(64−169) ***	0.000
MELD score	7.72(7.16−8.55)	10.81(8.61−13.08) ***	0.000

[a]Note: Values are expressed as medians (interquartile ranges, IQRs) or frequencies. *P* values were calculated from nonparametric Kruskal−Wallis test for continuous variables, Fisher's exact test for categorical variables for multiple comparisons correction and adjusted by the false discovery rate (FDR) method. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ when compared to CHB. Abbreviations: CHB, hepatitis B; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ALT, alanine transaminase; AST, aspartate transaminase; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; TBA, total bile acid; CREA, creatinine; BUN, urea nitrogen; FFA, free fatty acid; PT, prothrombin time; PTR, prothrombin time ratio; APTT, activated partial thromboplastin time; FIB, fibrinogen; INR, international normalized ratio; AFP, alpha-fetoprotein; CEA, carcino embryonie antigen; MCH, mean corpuscular hemoglobin; MELD, model for end-stage liver disease; TP, total protein; ALB, albumin; PALB, prealbumin; LDH, lactate dehydrogenase; CHE, choline esterase; TC, cholesterol; TG, triglyceride; VLDL, very low density lipoprotein; ApoA1, apolipoprotein A1; RBC, red blood cell; HCT, hematocrit; HGB, hemoglobin; PLT, platelet; GLB, globulin; UA, uric acid; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein; ApoB, apolipoprotein B; GLU, glucose; TT, thrombin time; WBC, white blood cell; MCHC, mean corpuscular hemoglobin concentration ; MPV, mean platelet volume; HBV-DNA, hepatitis B virus desoxyribonucleic acid.

metabolomics based on ultraperformance liquid chromatography coupled with triple quadrupole mass spectrometry (UPLC-MS/MS) to examine the distributions and alterations of serum amino acids in chronic hepatitis B (CHB) patients and HBV-associated HCC patients, in order to understand the metabolic differences between hepatitis and HCC. We found that the levels of several amino acids as well as several amino acid ratios were altered in those patients.

MATERIALS AND METHODS

Subjects. 136 patients with CHB and 93 patients with HCC were recruited from Longhua Hospital, Shanghai University of Traditional Chinese Medicine (Shanghai, China), during August of 2012 to June of 2014. The diseases were confirmed by biochemical, virological, imaging, and pathological examinations according to the diagnostic criteria of CHB and carcinoma.[10,11] The detailed criteria of inclusion and exclusion were shown in our former research.[8] The protocols as well as the informed consent for participants were approved by the Ethics Committee of Shanghai University of Traditional Chinese Medicine.

Clinical Data Collection. The fasting blood samples were carefully obtained from each participant, and we allowed clotting for 2 h. Then, samples were centrifuged at 3000*g* for 10 min, and serum was separated and aliquoted. All subjects underwent conventional clinical, hematological, biochemical, and serological evaluations. The HBV desoxyribonucleic acid (HBV-DNA) viral load was detected by Shanghai Adicon Clinical laboratories Inc. The Model for End-Stage Liver Disease (MELD) Score was required based on the equation: MELD Score = 9.6 × ln[creatinine (CREA)] (mg/dl) + 3.8 × ln[total bilirubin (TBIL)] (mg/dl) + 11.2 × ln[international normalized ratio (INR)] + 6.4 × 1.[12]

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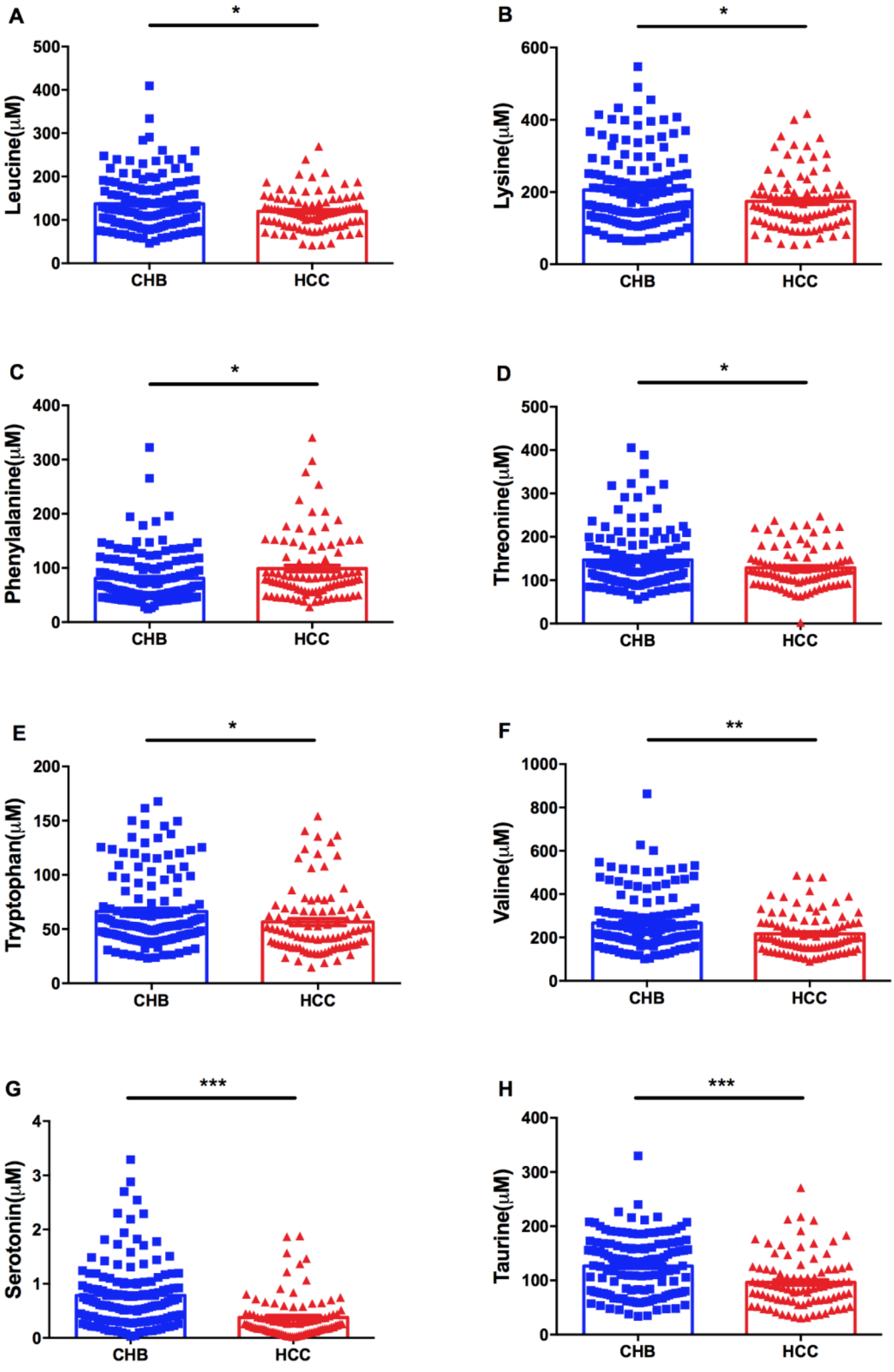

Figure 1. Individual data of leucine, lysine, phenylalanine, threonine, tryptophan, valine, serotonin, and taurine concentrations in serum for each patient in CHB- and HBV-associated HCC patients. The figure represents the individual data for each patient to show the distribution of values in each group. *, $p < 0.05$; **, $p < 0.01$; **, $p < 0.001$ when compared to CHB. CHB, hepatitis B; HBV, hepatitis B virus; HCC, hepatocellular carcinoma.

Serum Amino Acids Metabolomics Analysis. Ten microliter serum samples were used with a Waters Acquity UPLC instrument coupled with mass spectrometry (MS/MS) and quantified by the Absolute IDQ kit (Biocrates Life Sciences AG), and the details were described previously.[8] Two amino acids, including Ac-ornithine and carnosine, in the kits

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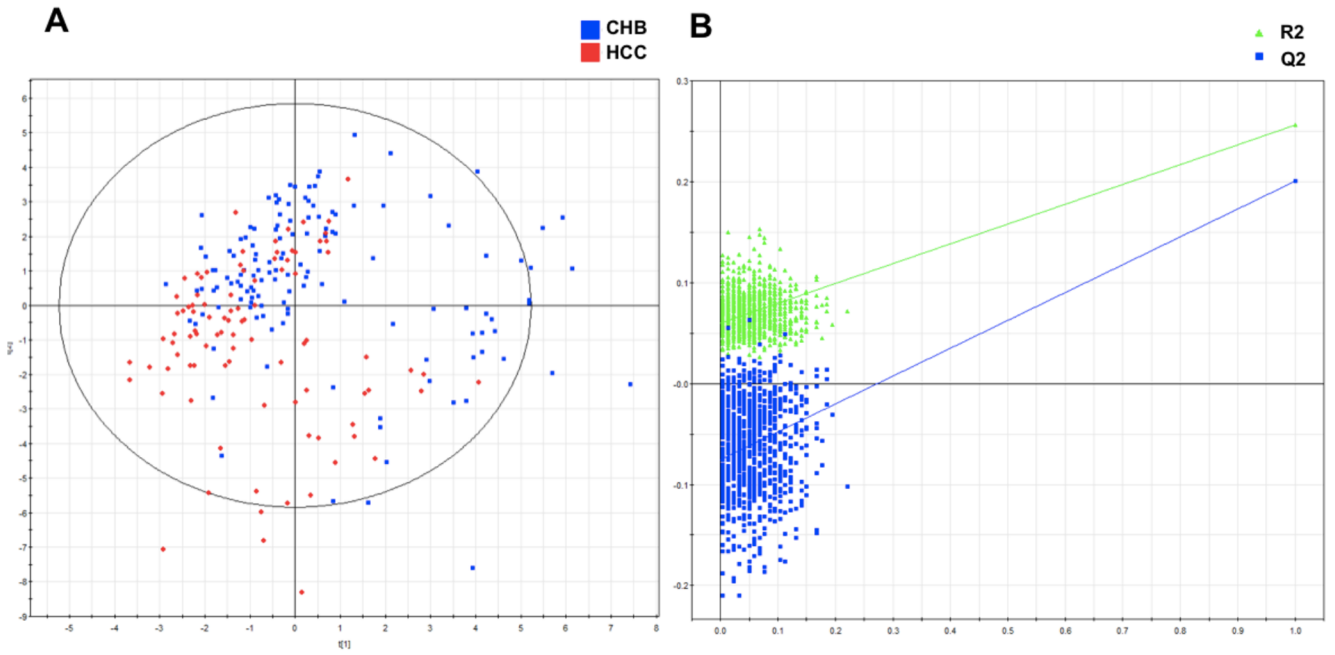

Figure 2. PLS-DA score plots show a clear separation between CHB- and HBV-associated HCC patients based on 40 variables. (A) 2D PLS-DA scores plot: R2X = 0.427, R2Y =0.256, Q2 = 0.201. (B) Permutation analysis: R2 = 0.06, Q2 = −0.09.

cannot be well-separated with the current method, and thus we excluded these two amino acids; finally, 40 amino acids were detected.

Statistical Analysis. Data are shown as medians (25−75th interquartile ranges, IQRs) or frequencies. The non-parametric Kruskal−Wallis test or Fisher's exact test was used to compare clinical indicators within groups with SPSS Statistics 17 (SPSS Inc.). The *P* value was adjusted using the false discovery rate (FDR) method, and $P < 0.05$ was considered statistically significant.

Multivariate profile-wide predictive models were established using principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) using SIMCA-P 11.5 (Umetrics). R2X, R2Y, and Q2Y values and a permutation test were performed to assess the reliability of models. A multiple logistic regression analysis was used to access the effect of gender, age, and body mass index (BMI) on the performance of representative amino acid levels. Then the differences of the above metabolites between subgroups within two groups based on the classification of different Child-Pugh Class (A, B, C), MELD score (≤8.99, >8.99), different stages (compensated, decompensated stage), and HBV-DNA ($<1 \times 10^{03}$, $\geq 1 \times 10^{03}$) were further analyzed.

RESULTS

Demographics and Clinical Characteristics. The clinical characters of patients are detailed described in Table 1. A total of 229 patients, of whom 136 had CHB and 93 had HCC, was included in this study. All patients were hepatitis B surface antigen (HBsAg)-positive. CHB patients were comprised of 62.5% men (85/51), whereas HCC patients were 84.9% men (79/14). The median age of CHB patients was 41 years old, and the median BMI was 23.66 kg/m^2, compared to 56 years and 22.31 kg/m^2 for HCC patients. Higher serum levels of TBIL, direct bilirubin (DBIL), indirect bilirubin (IBIL), alkaline phosphatase (ALP), γ-glutamyl transferase (GGT), total bile acid (TBA), CREA, urea nitrogen (BUN), free fatty acid (FFA), prothrombin time (PT), prothrombin time ratio (PTR), activated partial thromboplastin time (APTT), fibrinogen (FIB), INR, α-fetoprotein (AFP), carcino embryonie antigen (CEA), mean corpuscular hemoglobin (MCH), and MELD score were found in HCC patients than those in CHB.

However, serum levels of total protein (TP), albumin (ALB), prealbumin (PALB), lactate dehydrogenase (LDH), choline esterase (CHE), cholesterol (TC), triglyceride (TG), very low density lipoprotein (VLDL), apolipoprotein A1 (ApoA1), red blood cell (RBC), hematocrit (HCT), hemoglobin (HGB), and platelet (PLT) as well as the virus load of HBV-DNA were lower in HCC than those in CHB. No significant differences were observed in the levels of serum alanine transaminase (ALT), aspartate transaminase (AST), globulin (GLB), uric acid (UA), high density lipoprotein (HDL-C), low density lipoprotein (LDL-C), apolipoprotein B (Apo B), glucose (GLU), thrombin time (TT), white blood cell (WBC), mean corpuscular hemoglobin concentration (MCHC), and mean platelet volume (MPV) between the two groups.

Serum Concentrations of Amino Acids. All of the metabolomics analysis was performed on the population from 229 HBV-related diseases with fasting serum concentrations of 40 amino acids. Details were shown in the Supporting Information, Table S1. It was interesting that HCC patients showed more significant decreased serum levels of leucine, lysine, threonine, tryptophan, valine, serotonin, and taurine than CHB patients. In contrast, the concentration of phenylalanine, one of the aromatic amino acids (AAA), was significantly increased. Scatter diagrams showed differences of the above eight amino acids between CHB and HCC patients (Figure 1).

Interestingly, seven of eight amino acids were significantly different between groups when adjusting for gender, age, and BMI, except phenylalanine according to a logistic regression analysis (Supporting Information, Table S2).

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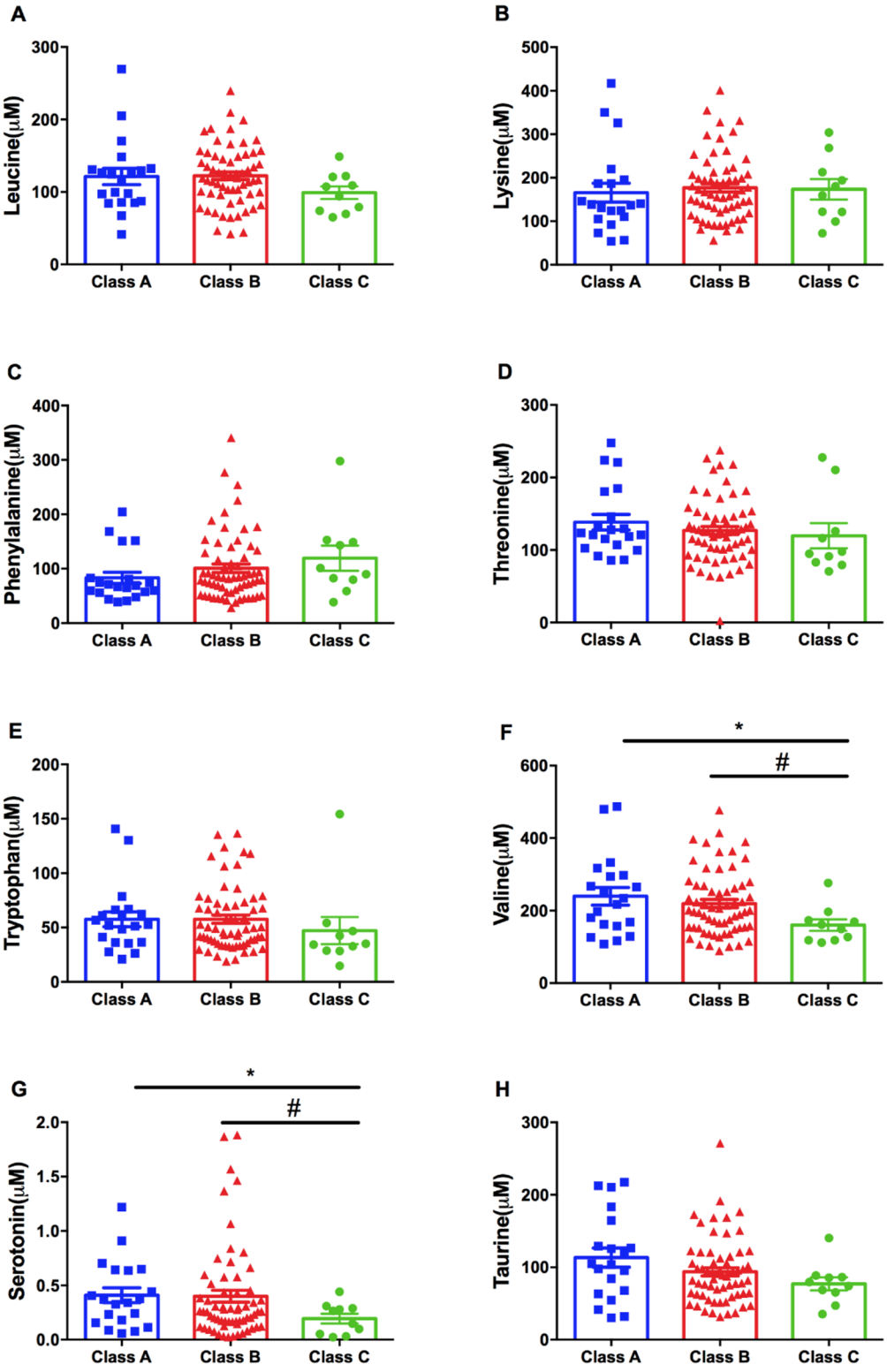

Figure 3. Serum valine and serotonin levels decreased more in Class C than Class A and Class B in patients with HBV-associated HCC. (A) Leucine, (B) lysine, (C) phenylalanine, (D) threonine, (E) tryptophan, (F) valine, (G) serotonin, (H) taurine. *P* values were calculated from a nonparametric Kruskal–Wallis test and adjusted by the FDR method. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ when compared to Class A. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.01$when compared to Class B. Child-Pugh Class, A = 5–6, B = 7–9, C = 10–15, based on hepatic encephalopathy, ascites, bilirubin, albumin, and prothrombin time (seconds).

To further sift out the effect of gender on serum levels of amino acids, a subgroup analysis was performed consequently. We compared not only the differences of 40 amino acids between female and male patients with CHB or HCC but also the differences between CHB and HCC in female and male patients. Overall, it is consistent with the results of logistic

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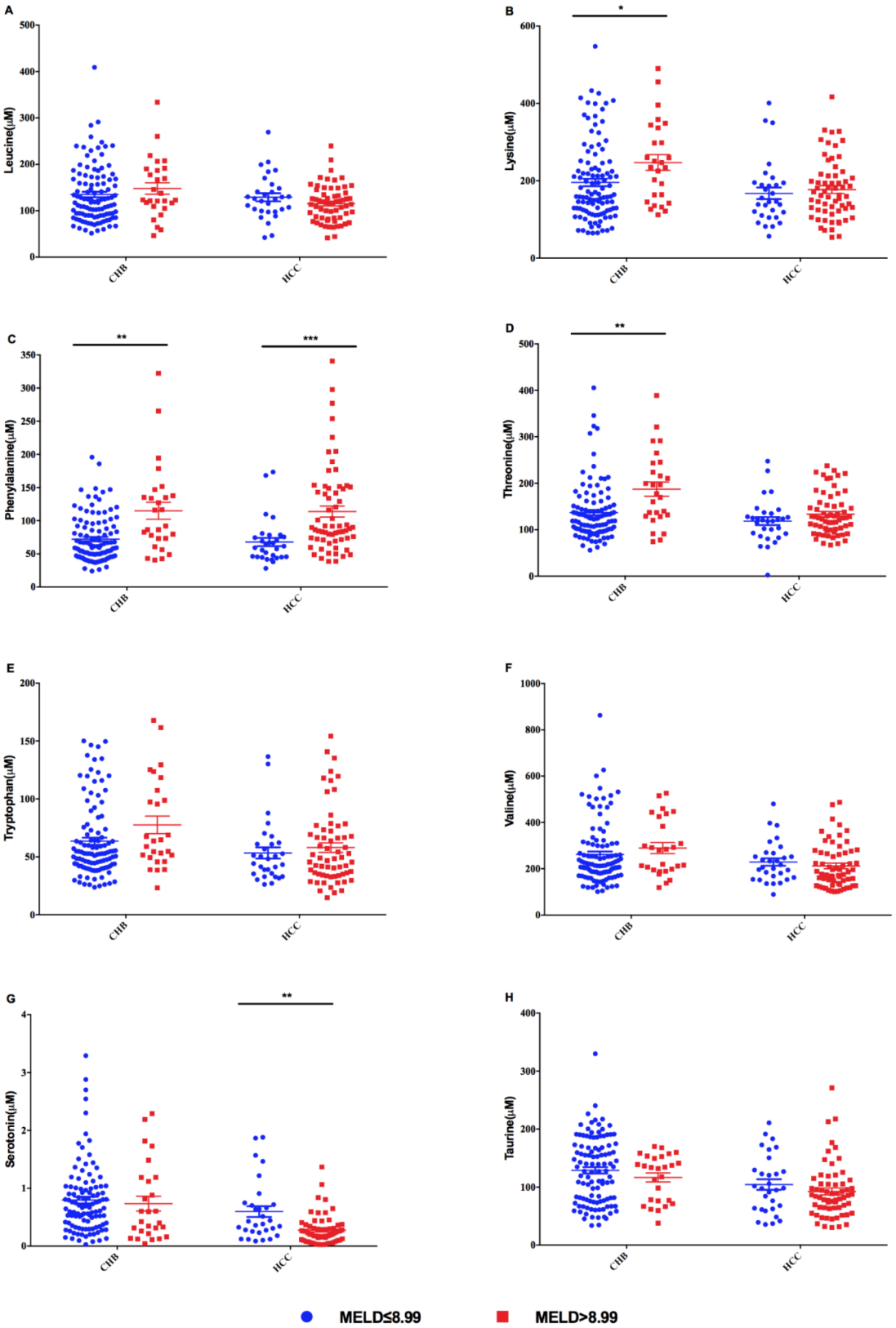

Figure 4. Serum phenylalanine is associated with different MELD score class in patients with CHB and HBV-associated HCC. (A) Leucine, (B) lysine, (C) phenylalanine, (D) threonine, (E) tryptophan, (F) valine, (G) serotonin, (H) taurine. *P* values were calculated from a non-parametric Kruskal–Wallis test and adjusted by the FDR method. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ when compared to MELD ≤ 8.99. Classification and equation: MELD score = 9.6 × ln(CREA) (mg/dl) + 3.8 × ln(TBIL) (mg/dl) + 11.2 × ln(INR) + 6.4 × 1.

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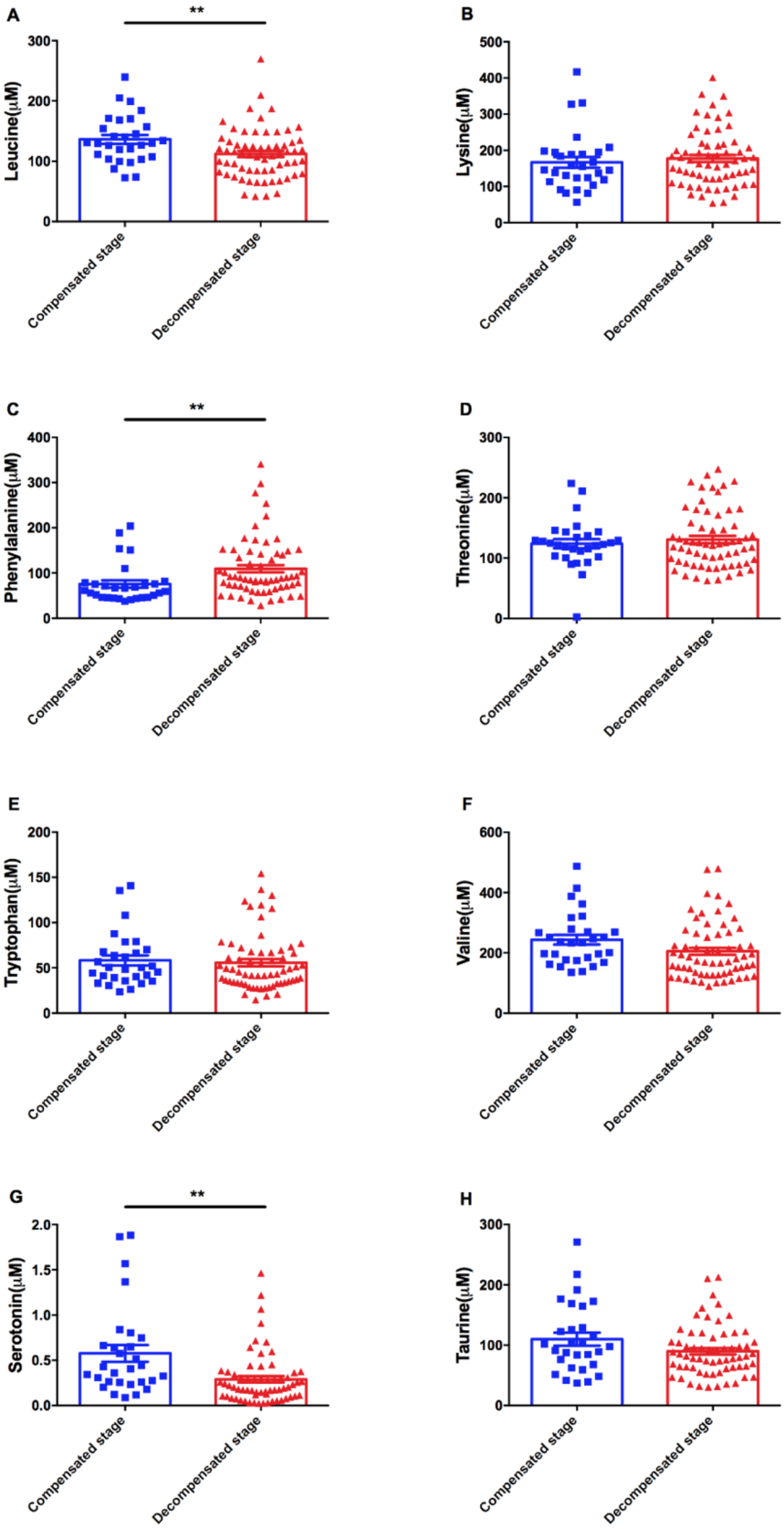

Figure 5. Serum leucine, phenylalanine, and serotonin are associated with different compensated stages in patients with HBV-associated HCC. (A) Leucine, (B) lysine, (C) phenylalanine, (D) threonine, (E) tryptophan, (F) valine, (G) serotonin, (H) taurine. *P* values were calculated from a nonparametric Kruskal−Wallis test and adjusted by the FDR method. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ when compared to the compensated stage.

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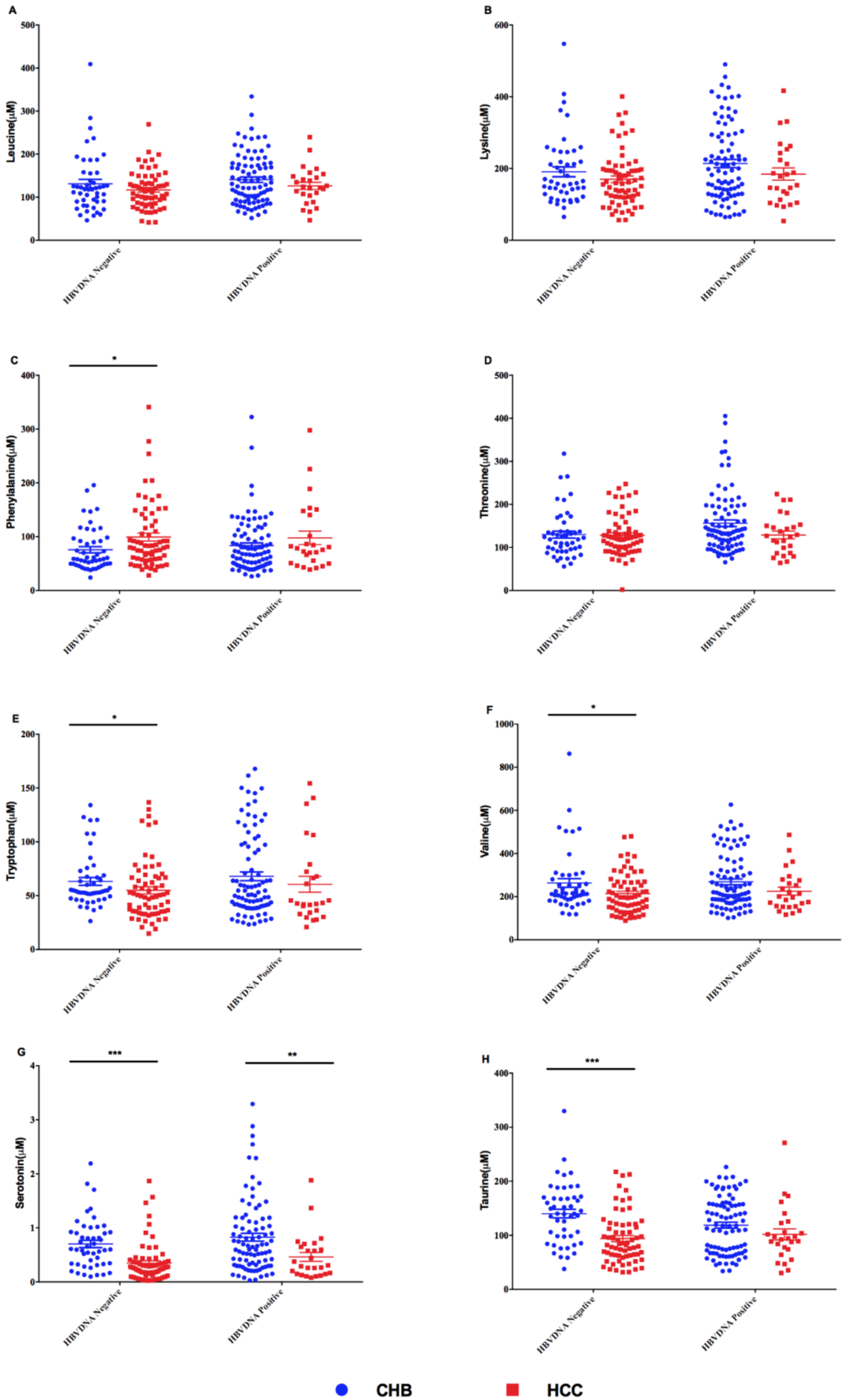

Figure 6. Serum serotonin is still significantly different between CHB- and HBV-associated HCC in both HBV-DNA negative and HBV-DNA positive groups. (A) Leucine, (B) lysine, (C) phenylalanine, (D) threonine, (E) tryptophan, (F) valine, (G) serotonin, (H) taurine. *P* values were calculated from a non-parametric Kruskal–Wallis test and adjusted by the FDR method. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ when compared to CHB. HBV DNA (log IU/mL). HBV-DNA Class, Negative = HBV-DNA $< 1 \times 10^{03}$, Positive = HBV-DNA $\geq 1 \times 10^{03}$.

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regression analysis, except phenylalanine; the other seven serum amino acids still had significant differences between patients with CHB and HCC no matter whether the patients were female or male. Details were shown in the Supporting Information, Table S3.

A subgroup analysis among different age stages was also performed. We classified patients into three age stages, that is, Age ≤ 40, 40 < Age ≤ 60, an 60 < Age. We not only compared the differences of 40 amino acids among different age stages patients with CHB or HCC but also the differences between CHB and HCC in patients at age stages. It was found that, except leucine and phenylalanine, the other six serum amino acids still had significant differences between patients with CHB and HCC no matter the age stage (Age ≤ 40, 40 < Age ≤ 60, or 60 < Age). Details were shown in the Supporting Information, Table S4.

PLS-DA score plots showed a clear separation between CHB and HCC based on 40 metabolites: R2X = 0.427, R2Y = 0.256, Q2 = 0.201; a permutation test showed the reliability: R2 = 0.060, Q2 = −0.075 (Figure 2, Supporting Information Figure S1). Combined with the results of variable influence on projection (VIP) value >1, it was shown that the contribution of leucine, lysine, phenylalanine, tryptophan, valine, serotonin, and taurine for the classification was 1.112, 1.047, 1.942, 1.103, 1.303, 2.206, and 1.705, respectively, except threonine was 0.953 (Supporting Information Table S5). We further performed a PLS-DA analysis between CHB and HCC based on eight differential metabolites. A clear separation was shown between CHB and HCC based on eight differential metabolites from PLS-DA score plots: R2X = 0.796, R2Y = 0.255, Q2 = 0.202; permutation test showed the reliability: R2 = 0.0196, Q2 = −0.0914 (Supporting Information Figure S2).

Serum Valine and Serotonin Are Associated with Child-Pugh Class in HCC Patients. A Child-Pugh score is the most common index to evaluate the severity of liver disease. We then determined the relationship between the Child-Pugh Class and metabolites according to the Child-Pugh score of A (5−6), B (7−9), and C (10−15). It was found that serum ALB was lower; however, serum TBIL was higher in Class C patients than those in Class A and Class B (Supporting Information Table S6). Among the above selected eight metabolites, lower serum valine and serotonin levels were found in Class C than Class A and Class B in HCC patients (Figure 3, Supporting Information Table S6).

We further performed a Spearman correlation analysis between serum differential amino acids and different variables including serum ALB and TBIL in HBV-associated HCC patients (Supporting Information Table S7). It was shown that serum phenyalanine was positively correlated with a Child-Pugh score (correlation coefficient (Corr) = 0.323); however, serum serotonin and taurine were negatively correlated with a Child-Pugh score (Corr = −0.291, −0.246), respectively. Serum phenyalanine was positively correlated with serum TBIL (Corr = 0.383); however, serum serotonin was negatively correlated with serum TBIL (Corr = −0.352). Serum phenyalanine was negatively correlated with serum ALB (Corr = −0.218); however, serum levels of leucine, tryptophan, valine, serotonin, and taurine were positively correlated with serum ALB (Corr = 0.248, 0.318, 0.36, 0.442, and 0.374, respectively).

Serum Phenylalanine Is Associated with a Different MELD Score Class in Patients with CHB or HCC. The MELD score is a common clinical factor to reflect the degrees of liver damage. We then classified patients with CHB or HCC according to a MELD score (≤8.99 or >8.99) in order to analyze the alteration of amino acids in different MELD scores. Finally, we found that, accompanied by the higher MELD score, the serum level of phenylalanine was increased not only in CHB patients but also in HBV-associated HCC patients (Figure 4, Supporting Information Table S8).

Serum Leucine, Phenylalanine, and Serotonin Are Associated with Different Compensated Stages in HCC Patients. We then studied the difference of serum AA levels in different stages of carcinoma patients. The serum level of phenylalanine increased more noticeably in the decompensated stage than in the compensated stage, while serum leucine and serotonin levels significantly decreased (Figure 5, Supporting Information Table S9).

Serum Serotonin Is Still Significantly Different between CHB and HCC in both HBV-DNA Negative and HBV-DNA Positive Groups. We classified patients into an HBV-DNA negative group ($<1 \times 10^{03}$) and an HBV-DNA positive group ($\geq 1 \times 10^{03}$) based on their HBV-DNA level in CHB and HCC. It was shown that serotonin was still significantly different between CHB- and HBV-associated HCC both in the HBV-DNA negative group and in the positive group (Figure 6, Supporting Table S10).

Several Ratios of Amino Acids Are Different between CHB and HCC Patients. In order to further know the alteration of amino acids, we analyzed several ratios of amino acids including tryptophan ratio, tyrosine ratio, branched-chain amino acids (BCAAs)/AAA ratio, BCAAs/tyrosine ratio (BTR), Fischer's ratio (BCAAs/tyrosine + phenylalanine), kynurenine-to-tryptophan ratio (KTR), and serotonin-to-tryptophan ratio (STR). It was shown that the tryptophan ratio, BCAAs/AAA ratio, BTR, Fischer's ratio, and STR significantly decreased in HCC patients more than in CHB patients, while the tyrosine ratio and KTR increased (Supporting Information Figure S3, Table S1). Interestingly, the above amino acid ratios still had significant differences between groups when adjusting for gender, age, and BMI according to a logistic regression analysis (Supporting Information Table S2).

In order to further sift out the effect of gender and age on serum amino acid ratios, a subgroup analysis was performed consequently. It was found that, except the tryptophan ratio, the other six serum amino acid ratios still had significant differences between patients with CHB and HCC no matter whether the patients were female or male (Supporting Information Table S3).

It was found that all seven serum amino acid ratios still had significant differences between patients with CHB- and HBV-associated HCC no matter the age stage (Age ≤ 40, 40 < Age ≤ 60, or 60 < Age) (Supporting Information Table S4).

PLS-DA score plots showed a clear separation between CHB- and HBV-associated HCC based on seven serum amino acid ratios: R2X = 0.812, R2Y = 0.263, and Q2 = 0.213; a permutation test showed the reliability: R2 = 0.011, Q2 = −0.084 (Supporting Figure S4).

Combined with the results of the VIP value greater than 1, it was shown that the tryptophan ratio, tyrosine ratio, BCAAs/AAA ratio, BTR, Fischer's ratio, KTR, and STR for the classification were 0.789, 1.067, 1.002, 1.121, 1.141, 0.894, and 0.938, respectively (Supporting Information Table S5).

We further performed the PLS-DA model based on 40 serum amino acid and 7 amino acid ratios, and it was shown

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that there was a clear separation between CHB and HCC: R2X = 0.491, R2Y = 0.345, Q2 = 0.237; a permutation test showed the reliability: R2= 0.090, Q2 = −0.015 (Supporting Information Figure S5). It was shown that leucine, lysine, phenylalanine, threonine, tryptophan, valine, serotonin, taurine, tryptophan ratio, tyrosine ratio, BCAAs/AAA ratio, BTR, Fischer's ratio, KTR, and STR for the classification were 0.991, 1.203, 1.071, 1.412, 0.872, 1.132, 1.793, 1.441, 1.110, 1.681, 1.849, 2.031, 2.105, 1.416, and 1.650, respectively (Supporting Information Table S5).

KTR increased in Class C more than in Class A and Class B, while the BCAAs/AAA ratio and Fischer's ratio decreased more in Class C than Class A in HCC patients (Supporting Information Figure S6, Table S11). It was found that the BCAAs/AAA ratio, BTR, Fischer's ratio, and STR decreased more in patients with a MELD score that was greater than 8.99 than those with a MELD score less than or equal to 8.99 in both CHB and HCC patients, and the tyrosine ratio increased in HCC patients (Supporting Information Figure S7, Table S12). The BCAAs/AAA ratio, BTR, Fischer's ratio, and STR decreased more in patients at a decompensated stage than those at a compensated stage in HCC patients (Supporting Information Figure S8, Table S13). It was shown that the BCAAs/AAA ratio, BTR, Fischer's ratio, and STR decreased, while the tyrosine ratio and KTR increased more in HCC patients than CHB both in the HBV-DNA negative group and in the positive group; however, a decreased tryptophan ratio between these two groups was found only in the HBV-DNA negative group (Supporting Information Figure S9, Table S14).

DISCUSSION

Targeted metabolomics based on the UPLC-MS/MS technique has been applied in many studies.[13−16] As we know, when operating in target metabolomics, the mass spectrometer is more sensitive and obtains ion data of specific predetermined mass-to-charge ratios (M/Z) and their specific fragments. Although the targeted method generates a narrower metabolome view, researchers are more confident in the output results, because UPLC-MS/MS could identity the signals and the method can be fully validated; thus, absolute quantification is possible. In the present study, we used a targeted metabolomics approach based on UPLC-MS/MS in order to analyze the distribution and composition of differentially regulated amino acids in serum from CHB- and HBV-associated HCC patients. The results demonstrated that amino acid metabolism was considered to be associated with the development of HCC.

Altered Amino Acids Involved in the Progression from CHB to HCC. We found an increased serum level of phenylalanine and decreased serum levels of serotonin, tryptophan, threonine, and taurine, the BCAAs including leucine and valine more in HCC patients than in CHB patients (Figure 1, Figure 2, Supporting Information Table S1). Serum valine and serotonin were more significantly decreased in Class C than Class A and Class B in HCC patients (Figure 3, Supporting Information Table S6). Accompanied with the higher MELD score, the serum level of phenylalanine was increased in both CHB and HCC (Figure 4, Supporting Information Table S8). Different stages showed different serum levels of leucine, phenylalanine, and serotonin in HCC patients (Figure 5, Supporting Information Table S9). The serum level of serotonin was still significantly different between CHB and HCC either in the HBV-DNA negative group or in the HBV-DNA positive group (Figure 6, Supporting Information Table S10).

Tryptophan and Serotonin. Tryptophan and its metabolites including kynurenine and serotonin play critical roles in various cellular growths.[17] In this study the serum levels of tryptophan and serotonin decreased more in HCC patients than in CHB patients. Furthermore, along with the severity of the Child-Pugh class, MELD score, and compensated stage, the serum serotonin level decreased significantly in patients with HCC. Differences between groups were found not only in the HBV-DNA-positive group but also in the negative group, which showed that the difference of the serum serotonin level between hepatitis and carcinoma was not affected by HBV. Our results were similar to those of the study that showed that serum serotonin can be a promising biomarker to follow-up patients who are at risk of developing HCC and for an early diagnosis of HCC.[18] However, there are several opposite results to our study. Liu et al.[19] found that HCC patients had significantly higher levels of portal vein serum and HCC tissue of L-tryptophan than healthy controls; these higher levels were related to impaired liver function and poor survival. Another study showed that serotonin may be used to screen for HCC in chronic hepatitis C-related cirrhotic patients.[20]

BCAAs: Valine, Isoleucine, and Leucine. Several prospective clinical studies showed that BCAA can protect from the formation and recurrence of HCC,[21] and BCAA supplementation reduces the risk for HCC.[22,23] Serum valine and leucine levels were lower in HCC than CHB in the present study, which suggested the ability of the protection of BCAA decreased. Luo et al.[24] found that BCAAs or a leucine treatment suppressed cisplatin- or BCAA transaminase 1-mediated autophagy by activating a mammalian target of rapamycin signaling in cancer cells. However, several studies showed that serum valine and choline were increased,[25] and valine in tissue[26] was increased in HCC patients.

AAA: Phenylalanine. Phenylalanine in the peripheral blood was elevated in HCC patients compared with that in liver cirrhosis patients.[27] Liang et al.[28] concluded that age, plasma phenylalanine, and glutamine concentrations altogether offered a risk score that was correlated with subsequent HCC occurrence in liver cirrhosis patients. In the present study, an increased serum phenylalanine level suggested a more serious impaired liver function in HCC patients than in CHB patients, because the liver is the major organ where phenylalanine is mainly metabolized.

Lysine, Threonine, Taurine. Although many studies have shown serine/threonine-protein kinase played important roles in tumorigenesis,[29,30] the mechanism of the decreased serum levels of threonine and lysine in HCC patients was unclear until now.

Taurine is an amino acid containing sulfur. It was shown that dietary taurine may protect from liver injury in patients with chronic hepatitis.[31] Tu et al.[32] observed taurine inhibited cell proliferation and apoptosis of human HepG2 cells. Asmaa et al.[33] found that taurine inhibited HepG2 cell proliferation and that taurine was assumed to be a promising and effective antitumor therapy of HCC. The serum taurine level decreased more in carcinoma patients than hepatitis patients in the present study, which showed the decrease of protection of the liver. However, until now it was unclear about the exact mechanism of taurine in protecting the liver from tumorigenesis.

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Altered Amino Acid Ratios Involved in the Progression from CHB to HCC. The tryptophan ratio, that is, the ratio of tryptophan to phenylalanine, tyrosine, leucine, valine, and isoleucine, was used to measure the availability of tryptophan to synthesize serotonin. The tyrosine ratio, that is, the ratio of tyrosine to phenylalanine, tryptophan, leucine, valine, and isoleucine, was used to measure the availability of tyrosine to synthesize dopamine and norepinephrine. Several studies have focused on the ratio between BCAA and AAA as a key component in the etiology of hepatic encephalopathy.[34]

BTR is useful to access liver function. Tada et al.[35] found that intervention with BCAA improved survival in patients with HCC (n = 66) versus those without using BCAA (n = 249), and a low BTR was related to a poor prognosis in patients. The BTR was a prognostic indicator for early HCC and a predictive indicator for intrahepatic recurrence and survival based on 50 HCC patients at stage I/II.[36] Yuji et al.[37] found that the BTR was an essential pretreatment indicator on maintaining the efficacy of lenvatinib in patients with HCC. Hiraoka et al.[38] concluded a low BTR (≤4.4) may be an available prognostic indicator in early HCC patients.

Fischer's ratio, the ratio of BCAAs (leucine, isoleucine, valine) to AAAs (tyrosine, phenylalanine), is vital to evaluate liver function.[39] Altogether, the decrease of BCAA/AAA, Fischer's ratio, and BTR in the present study suggested a more severe injured liver function in patients with HCC than those with CHB.

The KTR can be used as a prognostic marker to present cancer invasiveness and progression.[40] The KTR can be used as blood biomarker in nonsmall cell lung cancer.[41] Stepien et al.[42] found that serum leucine, lysine, glutamine, and Fischer's ratio were negatively related to HCC risk; however, tyrosine, phenylalanine, and the phenylalanine/tyrosine ratio, kynurenine, KTR, glutamate, and glutamate/glutamine ratio were positively related to HCC risk.

There are very few studies about STR in cancer studies, mainly because kynurenine represents more than 95% of tryptophan-catabolizing pathways. However, there are still unclear mechanisms about the alteration of KTR and STR in the development of HCC.

There are still several limitations in this study. First, the influences of diet on the serum amino acid level still could not be excluded. Second, there are no normal control groups. Third, because this is a cross-sectional study, the longitudinal approach is therefore required to confirm the present finding. Furthermore, in order to better understand the association between the serum amino acid level and the development of HCC, experiments to study the mechanism with in vivo and in vitro models are also required.

CONCLUSION

In conclusion, from a panel of 40 serum amino acid metabolites, a distinct metabolite signature of some specific serum amino acids was found between CHB and HCC patients, which may help predict the development of HCC at an early stage.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00885.

Serum concentrations of amino acids and several amino acid ratios in patients. Results of multiple logistic regression on the performance of representative serum amino acid levels and amino acid ratios. Comparison of 8 serum metabolites and 7 amino acid ratios in female and male patients with CHB or HBV-associated HCC. Comparison of 8 serum metabolites and 7 amino acid ratios in patients with CHB or HBV-associated HCC at different age stages. VIP value of 40 amino acids, 7 amino acid ratios and 40 amino acids +7 amino acid ratios from PLS-DA models in patients with CHB or HBV-associated HCC. Serum valine and serotonin were decreased in Class C than Class A and Class B in patients with HBV-associated HCC. Correlation between serum amino acids and different variables in HBV-associated HCC. Serum phenylalanine is associated with different MELD Score class in Patients with CHB and HBV-associated HCC. Serum leucine, phenylalanine and serotonin are associated with different compensated stages in patients with HBV-associated HCC. Serum serotonin is still significantly different between CHB and HBV-associated HCC in both HBV-DNA Negative and HBV-DNA Positive groups. Serum amino acid ratios in Class A, Class B and Class C in patients with HBV-associated HCC. Serum amino acid ratios are associated with different MELD Score class in patients with CHB and HBV-associated HCC. Serum amino acid ratios are associated with different compensated stages in patients with HBV-associated HCC. Serum amino acid ratios are still significantly different between CHB and HBV-associated HCC in both HBV-DNA Negative and HBV-DNA Positive groups. PLS-DA score plots show clear separation between CHB and HBV-associated HCC patients based on 40 amino acids. PLS-DA score plots show clear separation between CHB and HBV-associated HCC patients based on 8 amino acids. Individual data of several serum amino acid ratios in CHB and HBV-associated HCC patients. PLS-DA score plots show clear separation between CHB and HBV-associated HCC patients based on 7 serum amino acid ratios. PLS-DA score plots show clear separation between CHB and HBV-associated HCC patients based on 40 serum amino acid and 7 amino acid ratios. Serum amino acid ratios in Class A, Class B and Class C in patients with HBV-associated HCC. Serum amino acid ratios are associated with different MELD Score class in patients with CHB and HBV-associated HCC. Serum amino acid ratios are associated with different compensated stages in patients with HBV-associated HCC. Serum amino acid ratios are still significantly different between CHB and HBV -associated HCC in both HBV-DNA Negative and HBV-DNA Positive groups (PDF)

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Author Contributions

G.J. and W.J. designed the research. T.W. collected clinical data. T.W., X.J.Z., and A.H.Z. measured metabolites. T.W., M.Y., H.J.X., and T.L.C. performed statistical analysis. T.W. wrote the paper. G.J. and W.J. supervised the study and revised the paper. All authors read and approved the final manuscript.

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Notes

The authors declare no competing financial interest.

Availability of data and materials. All data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate. The protocols were approved by the Ethics Committee of Shanghai University of Traditional Chinese Medicine. The informed consent for participants was obtained.

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■ ABBREVIATIONS

CHB, hepatitis B; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ALT, alanine transaminase; AST, aspartate transaminase; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; TBA, total bile acid; CREA, creatinine; BUN, urea nitrogen; FFA, free fatty acid; PT, prothrombin time; PTR, prothrombin time ratio; APTT, activated partial thromboplastin time; FIB, fibrinogen; INR, international normalized ratio; AFP, alpha-fetoprotein; CEA, carcino embryonie antigen; MCH, mean corpuscular hemoglobin; MELD, model for end-stage liver disease; TP, total protein; ALB, albumin; PALB, prealbumin; LDH, lactate dehydrogenase; CHE, choline esterase; TC, cholesterol; TG, triglyceride; VLDL, very low density lipoprotein; ApoA1, apolipoprotein A1; RBC, red blood cell; HCT, hematocrit; HGB, hemoglobin; PLT, platelet; GLB, globulin; UA, uric acid; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein; ApoB, apolipoprotein B; GLU, glucose; TT, thrombin time; WBC, white blood cell; MCHC, mean corpuscular hemoglobin concentration; MPV, mean platelet volume; HBV-DNA, hepatitis B virus desoxyribonucleic acid

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