

Metabolomics reveals metabolite profiles associated with clinical cure of chronic HBV infection under different drug interventions

Peng Peng¹, Huiping Xu², Xianghong Kong³, Junling Wang⁴, Wei Zhang⁵

Abstract

Objective: To identify differential metabolites associated with complete cure in chronic hepatitis B patients treated with various regimens of entecavir and pegylated interferon-alpha.

Methods: The retrospective, case-control study was conducted at the Mudan People's Hospital of Heze, Shandong, China, and comprised samples from the institutional laboratory department of hepatitis B virus patients enrolled between June 2, 2021, and December 30, 2023. The patients were grouped based on the treatment protocol and the evidence of complete cure. Groups A and B comprised patients who were and were not clinically cured after receiving 48 weeks of sequential entecavir and pegylated interferon-alpha. Group E patients received entecavir monotherapy for over 2 years without a clinical cure, and group W included untreated patients. Ultra-performance liquid chromatography-mass spectrometry and metabolomics databases were utilised to identify differential metabolites and networks indicative of clinical cure. Data was analysed using the R language Ropls package.

Results: Of the 50 patients, 13(26%) each were in Group A and Group B, while 12(24%) each were in Group E and Group W. The median age was similar across groups (Group A: 42 [40-47] years; Group B: 43 [40-49] years; Group E: 43.5 [39-48] years; Group W: 43 [40-48] years), and male patients predominated in each group (Group A: 10(77%); Group B: 10(77%); Group E: 9(75%); Group W: 9(75%)). A total of 52 differential metabolites showing altered abundance in Group A compared with the other groups were identified. Notably, 9,10-dihydroxy-12Z-octadecenoic acid, 12,13-dihydroxy-9Z-octadecenoic acid and kaempferide were common differential metabolites across all intergroup comparisons. Beyond these common metabolites, the top-ranked differential metabolites unique to each comparison included guanidoacetic acid, caffeine and uracil 5-carboxylate in Group A versus Group B; 3-ketosphingosine in Group A versus Group E; and L-2-hydroxyglutaric acid and 3,4-dihydroxybenzeneacetic acid in Group A versus Group W. The fold-change values were 1.28 for 9,10-DHOME, 1.12 for 12,13-DHOME and 1.33 for kaempferide, and guanidoacetic acid ranked first among the differential metabolites in Group A versus Group B (fold-change: 1.27, $p=2.56 \times 10^{-8}$). These metabolites were enriched in 42 metabolic pathways, including linoleic acid, caffeine and amino acid metabolism.

Conclusions: Kaempferide, 9,10-dihydroxy-12Z-octadecenoic acid and 12,13-dihydroxy-9Z-octadecenoic acid were common differential metabolites, indicating potential significance for achieving clinical cure in chronic hepatitis B virus infection. Guanidoacetic acid, caffeine, uracil 5-carboxylate, 3-ketosphingosine, L-2-hydroxyglutaric acid, and 3,4-dihydroxybenzeneacetic acid were the top three differential metabolites in each group beyond the common ones.

Keywords: Chronic HBV infection, Clinical cure/functional cure, Metabolomics, Ultra-performance liquid chromatography-mass spectrometry, UPLC-MS, Entecavir, ETV, Pegylated interferon alpha, Peg-IFN α . (JPMA 76: 1905; 2026) DOI: <https://doi.org/10.47391/JPMA.33476>

Introduction

The concept of pursuing a complete clinical cure for chronic hepatitis B virus (HBV) infection was first proposed by the Chinese guidelines for the prevention and treatment of chronic hepatitis B virus HBV.¹ In 2016, a consensus was reached on the feasibility of achieving a clinical cure for

HBV infection (functional cure).² The concept of clinical cure was further confirmed by the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASLD).^{3,4}

A meta-analysis demonstrated the effectiveness of the sequential treatment of nucleotide analogues combined with pegylated interferon-alpha (PGE-IFN α) for HBV.⁵ However, while this regimen is associated with a significant increase in the cure rate (from 3-7% to 31.34%) for certain patients, some cannot achieve hepatitis B surface antigen (HBsAg) seroconversion.⁶ Furthermore, some patients may be unaware of their HBV status or have never received any treatment for their HBV infection. Studies indicate that the inability to achieve a clinical cure may be influenced by various genetic, immune and nutritional factors.

^{1,4}Department of Liver Diseases, Mudan People's Hospital of Heze, Heze, China;

²Department of Medical Imaging, Mudan People's Hospital of Heze, Heze,

China; ³Department of Infectious Diseases, Mudan People's Hospital of Heze,

Heze, China; ⁵Department of Clinical Lab, Mudan People's Hospital of Heze, Heze, China.

Correspondence: Peng Peng. e-mail: conjasen@126.com

ORCID ID: 0009-0007-9160-9645

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Metabolomics is a new technology that enables the qualitative and quantitative analysis of metabolic levels of various systems and changes in regulatory pathways, and was successfully used to analyse pathogenic mechanisms of numerous infectious diseases. However, studies that use the metabolomics approach to investigate HBV are still scarce.

The current study was planned to use metabolomics to identify differential metabolites associated with complete cure in chronic HBV patients treated with various regimens of entecavir (ETV) and PGE-IFN α .

Materials and Methods

The retrospective, case-control study was conducted at the Mudan People's Hospital of Heze, Shandong, China, and comprised samples from the institutional laboratory of HBV patients enrolled between June 2, 2021, and December 30, 2023. Data was collected using purposive sampling method based on a pre-defined detailed inclusion and exclusion criteria (Table 1).

All the patients were subjected to a comprehensive routine blood examination. To confirm hepatitis B e-antigen (HBeAg)-negative stage of chronic HBV infection, quantitative assessment of HBV viral load, liver, kidney and thyroid functions, blood glucose, blood lipids, alpha-fetoprotein and anti-nuclear antibodies (ANA) were tested using a fully automated biochemical analyser (AU5821, Beckman, United States), fully automated chemiluminescence analyser (Wan200+, Yumaike, China), and real-time quantitative polymerase chain reaction (PCR) analyser (ABI7500, Thermo Fisher Scientific, USA). The patients were further examined by the Department of

Ultrasound Medicine, Radiology and Ophthalmology using colour Doppler diagnostic equipment, liver transient elastography, digital X-ray camera, and fundus examination to exclude relevant contraindications. None of the patients had taken any anti-HBV deoxiribonucleic acid (DNA) drugs, immunomodulatory drugs, or traditional Chinese medicine (TCM) for the preceding six months. Additionally, there

Table-1: Inclusion and exclusion criteria for Groups A, B, E and W.

Group	Inclusion Criteria	Exclusion Criteria
A	a. HBeAg-negative chronic HBV infection (2017 EASL Guidelines) b. Age 18-65, gender-neutral c. Regular ETV therapy >2 years with ongoing treatment d. Normal lab/imaging results (CBC, LFTs, TFTs, AFP, etc.) e. HBV DNA(-), HBeAg seroconversion, baseline HBsAg <1500 IU/mL f. Achieved HBsAg <0.05 IU/mL through ETV+PEG-IFN α -2b therapy (48 weeks)	a. HBV-related comorbidities (e.g., glomerulonephritis) b. HIV/hepatitis co-infection c. Other liver diseases d. Severe systemic disorders e. Pregnancy/lactation f. Recent non-ETV drugs/TCM g. PEG-IFN contraindications (psychosis, decompensated cirrhosis, etc.)
B	a-e. Same as Group A f. Failed to achieve HBsAg <0.05 IU/mL with same sequential therapy (HBsAg >0.05 IU/mL sustained post-treatment)	Same as Group A exclusion criteria
E	a-d. Same as Group A e. HBV DNA(-), HBeAg seroconversion, baseline HBsAg >1500 IU/mL (persistent >1500 IU/mL after 48-week ETV monotherapy)	Same as Group A exclusion criteria a-f.
W	a-b. Same as Group A c. No prior anti-HBV treatment d. Normal diagnostic tests e. HBV DNA(+), HBsAg+/HBeAg-/HBcAb+	a-e. Same as Group A exclusion criteria. f. No recent immunomodulators/TCM use

EASL: European Association for the Study of the Liver, CBC: Complete blood count, LFTs: Liver function tests, TFTs: Thyroid function tests, AFP: Alpha-fetoprotein; HBsAg: Hepatitis B surface antigen, HBeAg: Hepatitis B e antigen, HBcAb: Hepatitis B core antibody, HBV DNA: Hepatitis B virus deoxyribonucleic acid, TCM: Traditional Chinese medicine, ETV: Entecavir, Peg-IFN α : Pegylated interferon alpha.

Table-2: Shared Metabolites and Top three differential metabolites across intergroup comparisons.

Pathway name	Metabolites	KEGG	Formula	FC	Group/Rank
Linoleic acid metabolism	9,10-DHOME	C14828	C18H34O4	1.28	AvsB/19*; AvsE/1**; AvsW/7*
Linoleic acid metabolism	12,13-DHOME	C14829	C18H34O4	1.12	AvsB/27*; AvsE/3**; AvsW/1***
-	Kaempferide	C10098	C16H12O6	1.33	AvsB/24*; AvsE/7*; AvsW/6**
Glycine, serine and threonine metabolism	Guanidoacetic acid	C00581	C3H7N3O2	1.27	AvsB/1****
Arginine and proline metabolism	Guanidoacetic acid	C00581	C3H7N3O2	1.27	AvsB/1****
-	Uracil 5-carboxylate	C03030	C5H4N2O4	2.45	AvsB/2****
Caffeine metabolism	Caffeine	C07481	C8H10N4O2	9.5	AvsB/3***
-	3-Ketosphingosine	C06121	C18H35NO2	0.9	AvsE/2***
Lysine degradation	L-2-Hydroxyglutaric acid	C03196	C5H8O5	0.87	AvsW/2***
Tyrosine metabolism	3,4-Dihydroxybenzeneacetic acid	C05580	C5H8O5	1.05	AvsW/3***
Dopaminergic synapse	3,4-Dihydroxybenzeneacetic acid	C05580	C5H8O5	1.05	AvsW/3***
Alcoholism	3,4-Dihydroxybenzeneacetic acid	C05580	C5H8O5	1.05	AvsW/3***
Cocaine addiction	3,4-Dihydroxybenzeneacetic acid	C05580	C5H8O5	1.05	AvsW/3***
Amphetamine addiction	3,4-Dihydroxybenzeneacetic acid	C05580	C5H8O5	1.05	AvsW/3***

9,10-DHOME: 9,10-dihydroxy-12Z-octadecenoic acid, 12,13-DHOME: 12,13-dihydroxy-9Z-octadecenoic acid, AvsB: Group A versus Group B, AvsE: Group A versus Group E, AvsW: Group A versus Group W, FC: Fold change; 9,10-DHOME, 12,13-DHOME, and kaempferide were differential metabolites shared by the AvsB, AvsE, and AvsW groups; Group/Rank: The former part indicates the pairwise comparison group corresponding to the differential metabolite, and the latter part represents the ranking of the metabolite in the corresponding OPLS-DA model sorted in descending order of VIP values. The higher the ranking, the greater the contribution of the metabolite to the inter-group differences; Asterisks indicate the significance of the differential metabolites between the two groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

were no HBV infection-related complications or other systemic diseases.

HBV patients who achieved clinical cure after receiving sequential treatment of ETV combined with PGE-IFN α for 48 weeks comprised group A. Patients who did not achieve clinical cure with the same treatment method comprised group B. Patients who received ETV monotherapy for >2 years without clinical cure were assigned to group E, and untreated patients with chronic HBV infection comprised group W.

Approval was obtained from the institutional ethics review committee, and all patients had signed informed consent forms.

Serum was extracted from 4mL of fasting venous blood from all the patients and preserved at -80°C for subsequent analysis. The extraction reagents and equipment used in the metabolomic detection process were noted separately (Supplementary Tables 1-2).

Serum metabolites were analysed using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). The extraction and analytical procedures were based on established methodologies with tailored modifications.⁷⁻⁹

For liquid chromatography (LC) separation, a UPLC system (Thermo Vanquish, Thermo Fisher Scientific, USA), which

had been duly equipped (ACQUITY UPLC, HSS T3 column, 2.1×100mm, 1.8 μ m; Waters, USA), was used. The column temperature was maintained at 40°C with a flow rate of 0.3mL/min and an injection volume of 2 μ L. The mobile phases consisted of 0.1% formic acid in acetonitrile and 0.1% formic acid in water for positive ion mode, and acetonitrile and 5mM ammonium formate in water for negative ion mode, employing a gradient elution programme.

For mass spectrometry (MS) detection, a mass spectrometer (Thermo Orbitrap Exploris 120, Thermo Fisher Scientific, USA) with an electrospray ionisation (ESI) source was operated in both positive and negative ion modes. The full scan range was set at m/z 100-1000 with a resolution of 60,000. Data-dependent MS/MS acquisition was performed with a collision energy of 30% and a second-stage resolution of 15,000.

Data pre-processing was done by converting raw MS files to the mzXML file format using the MSConvert tool in the Proteowizard software package v3.0.8789. Peak detection, peak filtering, and peak alignment processes were performed using the XCMS software package,¹⁰ with bw=2, ppm=15, peakwidth=c (5,30), mzwid=0.015, mzdiff=0.01 and method="centWave" to obtain a list of quantified substances. Support vector regression correction based on quality control (QC) samples was used to eliminate

Supplementary Table 1: Main reagents and standards for metabolite detection.

Name	CAS	Purity	Brand
Methanol	67-56-1	≥99.0%	Thermo
H ₂ O	NA	NA	Millipore
2-chloro-L-phenylalanine (internal standard reference material)	103616-89-3	98%	Aladdin
Acetonitrile	75-05-8	≥99.9%	Thermo
Formic acid	64-18-6	LC-MS	grade TCI
Ammonium formate	540-69-2	≥99.9%	Sigma

(Loughborough, United Kingdom). Ultrapure water was generated using a Milli-Q system (Millipore, Bedford, United States). 2-Amino-3-(2-chloro-phenyl)-propionic acid was obtained from Aladdin (Shanghai, China). LC-MS grade acetonitrile (ACN) was purchased from Fisher Scientific (Loughborough, UK). Formic acid was obtained from TCI (Shanghai, China). Ammonium formate was obtained from Sigma-Aldrich (Shanghai, China).

Supplementary Table 2: Metabolite extraction and detection instruments.

Name	Brand	Model
Refrigerated centrifuge	Xiangyi	H1850-R
Vortex mixer	Kylin-bell	BE-2600
Vacuum concentrator	Eppendorf	5305
Filter membrane	Jinteng	0.22 μ m PTFE
Liquid chromatograph	Thermo	Vanquish
Mass spectrometer	Thermo	Orbitrap Exploris 120

High-speed centrifuge was obtained from Hunan Xiangyi Experiment Equipment Co., Ltd. (Hunan, China). Centrifugal vacuum evaporator was from Eppendorf China Ltd. (Shanghai, China). Vortex mixer was obtained from Haimen Kylin-bell Lab Instruments Co., Ltd. (Haimen, China). Microporous membrane filters (0.22 μ m) was purchased from Tianjin Jinteng Experiment Equipment Co., Ltd. (Tianjin, China).

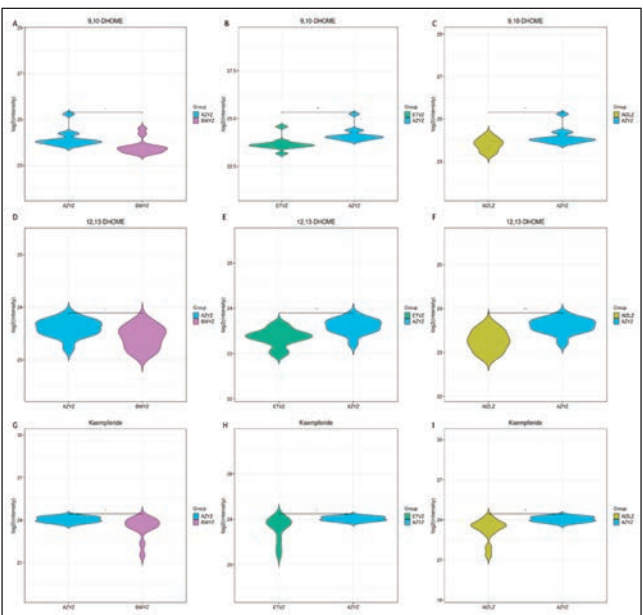


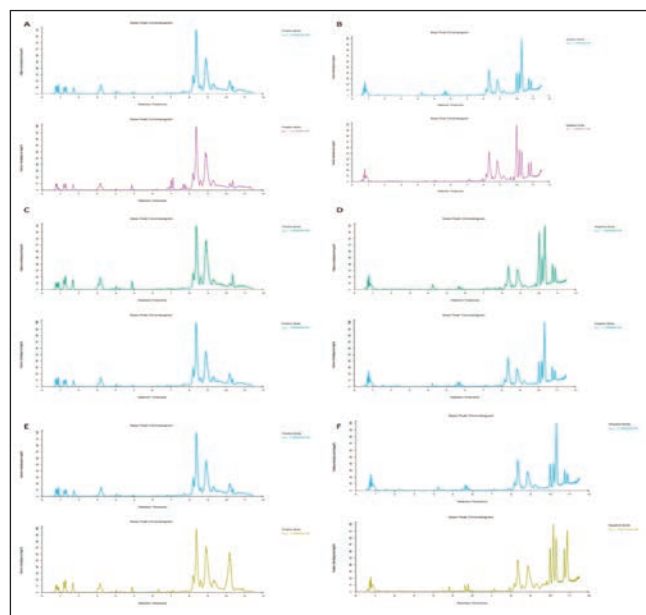
Figure-1: Violin plots of differential metabolites shared by the AvsB, AvsE and AvsW groups. Panels A-C: 9,10-DHOME; Panels D-F: 12,13-DHOME; Panels G-I: kaempferide. The horizontal axis represents different groups, and the vertical axis represents the range of metabolite quantitative values. Asterisks indicate the significance of the difference between the two groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

systematic errors. Substances with a coefficient of variation (CV) <30% (intra-assay CV) (9) in QC samples were retained for further analysis.

Base peak chromatogram (BPC) was used to display the intensity of the most intense peak at a given retention time (Supplementary Figure 1).

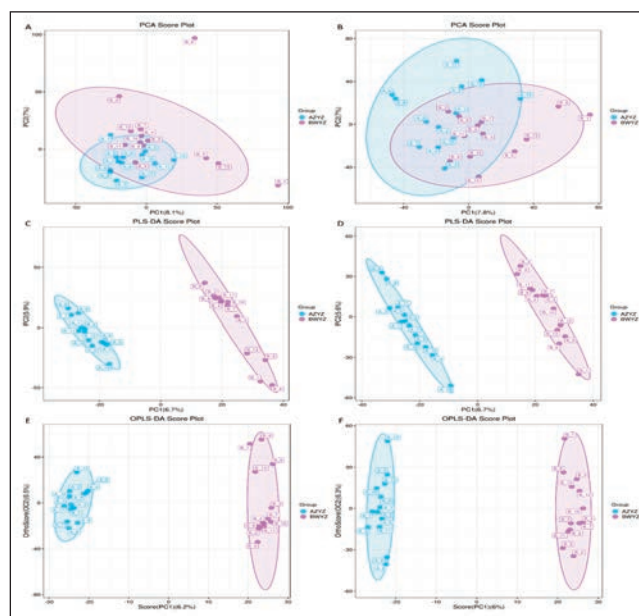
For QC, the potential feature peaks were required to have a relative standard deviation (RSD) within the QC samples or a CV not exceeding 30%. All feature peaks not meeting this requirement were removed.

After cluster analysis and hierarchical cluster analysis of the overall metabolites, multivariate statistical analysis was performed using the R language Ropls package.¹¹ Confounding factors, including age, gender and baseline ALT (alanine aminotransferase) levels were adjusted for in the subsequent subgroup analyses of the multivariate models. The methods included principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and orthogonal partial least squares-discriminant analysis (OPLS-DA). Before conducting multivariate statistical analysis on metabolomics data, appropriate weighting transformation, i.e., normalisation, was carried out. Data was log2-transformed to approximate a normal distribution and to facilitate the interpretation of fold changes (FCs). Adaptive conversion was used to process the data, yielding more intuitive results. Then, the PLS-DA

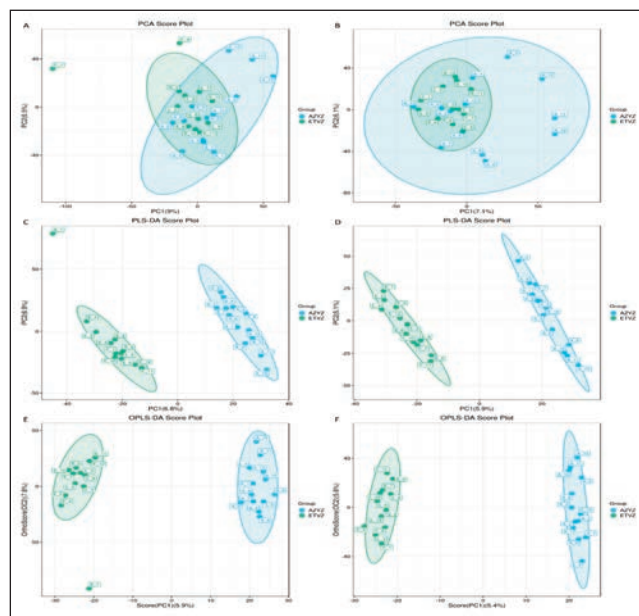


Supplementary Figure 1: Positive- and negative-ion mode base peak chromatograms (BPC) for the AvsB, AvsE and AvsW comparisons. Panels A-B: BPC for Groups A and B; Panels C-D: BPC for Groups A and E; Panels E-F: BPC for Groups A and W. The x-axis shows retention time and the y-axis shows ionic strength; the value in the upper right corner of each panel indicates the maximum ionic strength recorded for that sample.

model's first principal component's variable importance in the projection (VIP) value (>1 threshold) was used in combination with a PLS-DA permutation test and S-plot



Supplementary Figure 2: Positive- and negative-ion mode score plots for the AvsB comparison. Panels A-B: PCA (principal component analysis) score plot; Panels C-D: PLS-DA (partial least squares-discriminant analysis) score plot; Panels E-F: OPLS-DA (orthogonal partial least squares-discriminant analysis) score plot. The x-axis (PC1) and y-axis (PC2) represent the first and second principal component scores, respectively. Dots represent individual samples, circles denote the 95% confidence interval, and colours indicate group membership.



Supplementary Figure 3: Positive- and negative-ion mode score plots for the AvsE comparison. Panels A-B: PCA score plot; Panels C-D: PLS-DA score plot; Panels E-F: OPLS-DA score plot of AvsE group.

Supplementary Table 3: PCA, PLS-DA, OPLS-DA score plot model parameters.

Parameter		PCA			PLS-DA			OPLS-DA		
		AvsB	AvsE	AvsW	AvsB	AvsE	AvsW	AvsB	AvsE	AvsW
Positive ion	Pre	9	9	9	2	2	2	1+1+0	1+1+0	1+1+0
	R2X(cum)	0.504	0.504	0.526	0.126	0.137	0.167	0.126	0.137	0.167
	R2Y(cum)	NA	NA	NA	0.995	0.990	0.998	0.995	0.995	0.998
	Q2(cum)	NA	NA	NA	0.642	0.644	0.880	0.454	0.452	0.800
Negative ion	Pre	10	10	9	2	2	2	1+1+0	1+1+0	1+1+0
	R2X(cum)	0.504	0.504	0.526	0.122	0.110	0.120	0.122	0.110	0.120
	R2Y(cum)	NA	NA	NA	0.996	0.996	0.999	0.996	0.996	0.999
	Q2(cum)	NA	NA	NA	0.626	0.515	0.666	0.511	0.375	0.657

PCA: Principal Component Analysis; PLS-DA: Partial Least Squares-Discriminant Analysis; OPLS-DA: Orthogonal Partial Least Squares Discriminant Analysis; AvsB: Group A versus Group B; AvsE: Group A versus Group E; AvsW: Group A versus Group W; Pre: number of predictive components in the model; R2X(cum): cumulative proportion of variance in X (metabolite data) explained by the model; R2Y(cum): cumulative proportion of variance in Y (group classification) explained by the model; Q2(cum): cumulative predictive ability of the model estimated by cross-validation; NA: not applicable (PCA is an unsupervised method and does not generate R2Y or Q2 values).

analysis. Finally, differential expression metabolites were identified based on $p < 0.05$ and $VIP > 1.0$ from the OPLS-DA model. For the interpretation of results, FC was calculated as the ratio of the mean metabolite abundance in Group A (clinically cured) to that in the corresponding comparison groups (B, E or W). $FC > 1$ indicated up-regulation of the metabolite in Group A, while $FC < 1$ indicated down-regulation. Substance identification was conducted using spectrum databases, such as HMDB (Human Metabolome Database)¹² and KEGG (Kyoto Encyclopedia of Genes and Genomes),¹³ supplemented by an in-house metabolite standard database established by Nomi Biotech Corporation for comparison and searches.

Results

Of the 50 patients, 13(26%) each were in Group A and Group B, while 12(24%) each were in Group E and Group W. The median age was similar across groups (Group A: 42 [40–47] years; Group B: 43 [40–49] years; Group E: 43.5 [39–48] years; Group W: 43 [40–48] years), and male patients predominated in each group (Group A: 10(77%); Group B: 10(77%); Group E: 9(75%); Group W: 9(75%)). A total of 52 differential metabolites were identified, and the overall distribution trend of the samples as well as score plots were noted (Supplementary Figures 2–4).

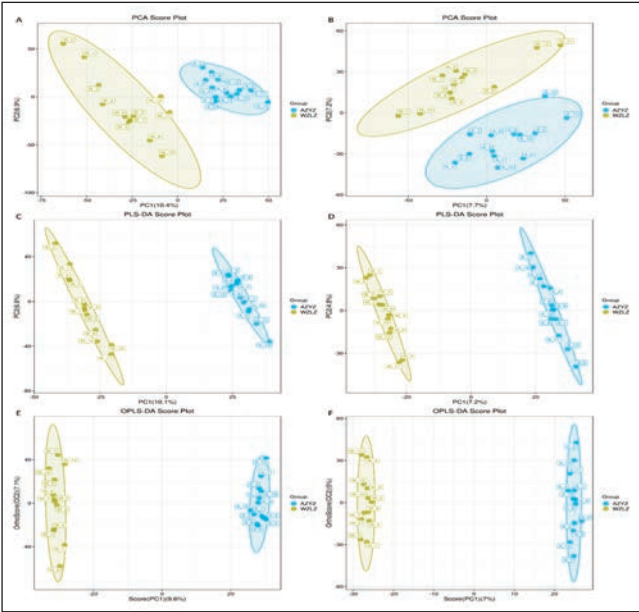
Notably, 9,10-dihydroxy-12Z-octadecenoic acid (9,10-DHOME), 12,13-dihydroxy-9Z-octadecenoic acid (12,13-DHOME) and kaempferide were common differential metabolites when Group A was compared with Group B, Group E and Group W, respectively (Table 2). Their detailed distributions across the groups as well as model evaluation parameters were detailed separately (Figure 1, Supplementary Table 3). The broader metabolic pathway alterations involving the shared metabolites reflected in the overall enrichment analysis of differential metabolites (Supplementary Figures 5–7).

In the specific comparison between Group A and Group B,

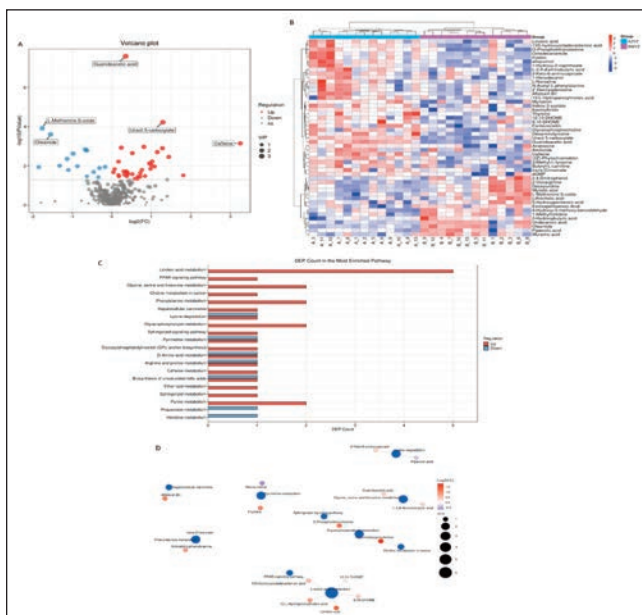
34 differential metabolites were identified (Supplementary Figure 5A). Their expression patterns across the groups (Supplementary Figure 5B), and the associated pathway enrichment results (Supplementary Figure 5C–D) were documented.

Comparing Group A with Group E revealed 13 differential metabolites (Supplementary Figure 6A). Metabolite clustering and pathway enrichment data was used for comparison (Supplementary Figure 6B–D).

The comparison between Group A and Group W uncovered 11 differential metabolites (Supplementary Figure 7A). Key differential metabolites were visualised in detail for comparison (Supplementary Figure 7B–D). The distribution of the top-ranked differential metabolites unique to each pairwise comparison, beyond those shared across all



Supplementary Figure 4: Positive- and negative-ion mode score plots for the AvsW comparison. Panels A–B: PCA score plot; Panels C–D: PLS-DA score plot; Panels E–F: OPLS-DA score plot of AvsW group.

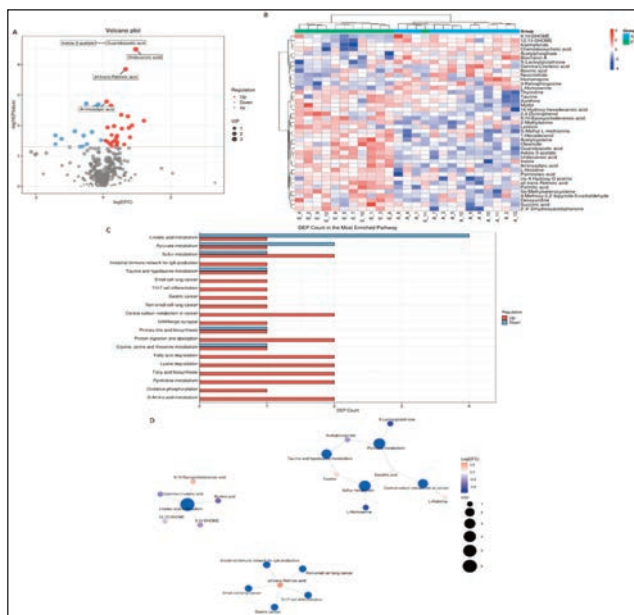


Supplementary Figure 5: A: Differential volcano plot. The X-axis represents the logarithm base 2 of the fold change in quantitative values of a metabolite between two samples; the Y-axis represents the negative logarithm base 10 of the P value, with each point in the graph representing a specific metabolite. The greater the absolute value on the X-axis, the larger the fold change in expression levels of a metabolite between the two samples. A larger value on the Y-axis indicates more significant differential expression and a more reliable selection of differentially expressed metabolites. The size of the points represents the VIP value, with red points indicating upregulated differences, blue points indicating downregulated differences, and grey points indicating metabolites that do not meet the differential filtering criteria. B: Differential molecule heat map. Columns represent samples, and rows represent metabolites. The hierarchical clustering tree on the left is for differential metabolite clustering, and the top is the sample clustering tree. The gradient colour represents the magnitude of quantitative values, with red indicating higher levels and blue indicating lower levels. C: KEGG pathway differential molecule count plot. The X-axis represents the number of differential metabolites, and the Y-axis represents different metabolic pathways. Red indicates the number of upregulated metabolites, while blue represents the number of downregulated metabolites. This analysis result is not available for comparisons of three groups or more. D: Network diagram. Blue points represent pathways, while the other points represent metabolites. The size of the pathway points indicates the number of metabolites connected to them: the more connections, the larger the point. The colour of the metabolite points indicates the magnitude of $\log_2(\text{FC})$ values: red indicates upregulation, blue indicates downregulation, and darker colours indicate more significant differences. Multiple group comparisons do not provide $\log_2(\text{FC})$ information for metabolites.

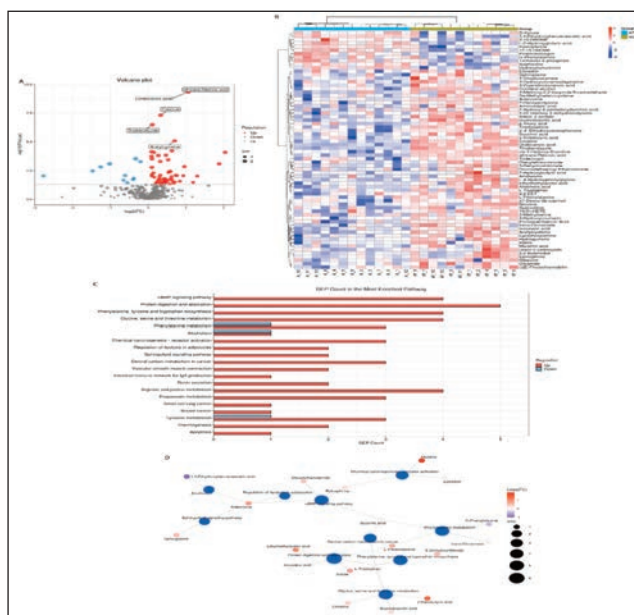
groups, was further illustrated (Supplementary Figure 8).

Discussion

The study, to our knowledge, is the first to analyse data on the clinical cure of chronic HBV infection using the UPLC-MS method. The analysis identified 52 differential metabolites that showed altered abundance in patients who were clinically cured after receiving 48 weeks of

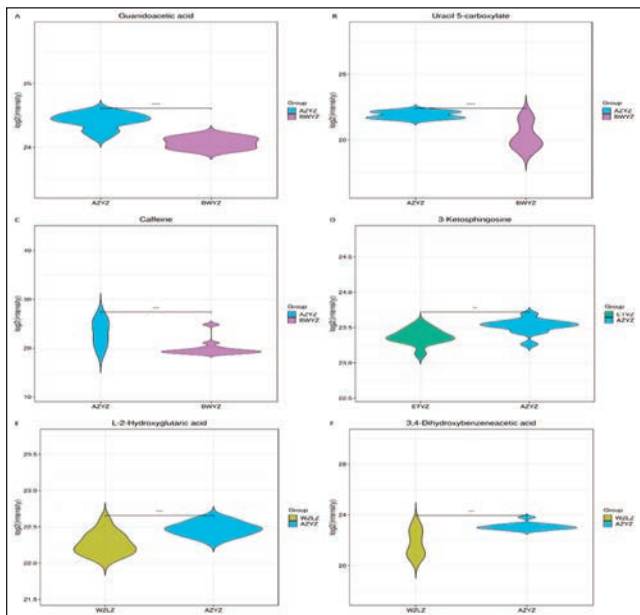


Supplementary Figure 6: A: Differential volcano plot; B: Differential molecule heat map; C: KEGG pathway differential molecule count plot; D: Network diagram. Panel definitions (axes, colour coding, VIP, $\log_2(\text{FC})$) are as described for Supplementary Figure 5.



Supplementary Figure 7: A: Differential volcano plot; B: Differential molecule heat map; C: KEGG pathway differential molecule count plot; D: Network diagram. Panel definitions (axes, colour coding, VIP, $\log_2(\text{FC})$) are as described for Supplementary Figure 5.

sequential ETV and PGE-IFN α , with 9,10-DHOME, 12,13-DHOME and kaempferide being more common in all HBV patients who underwent treatment compared to untreated patients. These metabolites showed potential significance in the clinical cure of chronic HBV infection as they were involved in 42 metabolic pathways, including linoleic acid



Supplementary Figure 8: Violin plots of the top three differential metabolites, in addition to those shared by the AvsB, AvsE, and AvsW groups. AvsB (ranking first): Guanidoacetic acid; AvsB (ranking second): Uracil 5-carboxylate; AvsB (ranking third): caffeine; AvsE (ranking second): 3-ketosphingosine; AvsW (ranking second): L-2-hydroxyglutaric acid; AvsW (ranking third): 3,4-dihydroxybenzoic acid. The first-ranked differential metabolite for the AvsE and AvsW comparisons (9,10-DHOME and 12,13-DHOME, respectively) is shared across all three comparisons and is shown separately in Figure 1.

metabolism, caffeine metabolism, and sphingolipid metabolism.

The treatment regimen of PEG-IFN α combined with nucleotide analogues remains the primary strategy for achieving clinical cure of chronic HBV infection.⁵ Nevertheless, the efficiency of this treatment strategy remains controversial, and the regimen is not recommended for patients under long-term NA suppression.³ Therefore, identifying treatment strategies to achieve a cure for HBV and developing new biomarkers is crucial.

The 9,10-DHOME and 12,13-DHOME are hydrolysis products of 9,10-EPOME and 12,13-EPOME (epoxyoctadecenoic acid), epoxy metabolites of linoleic acid. Linoleic acid is a precursor to arachidonic acid and plays a crucial role in human metabolism. Studies have shown that arachidonic acid expression is downregulated in the serum of patients with HBV replication,¹⁴ indicating that the high expression of linoleic acid, 9,10-DHOME, and 12,13-DHOME in the current Group A may participate in the clearance of HBV and HBsAg. Additionally, the results identified gamma-linolenic and bovinic acids that are involved in linoleic acid metabolism as differential metabolites that were enriched in patients who achieved a cure after sequential ETV and PGE-IFN α therapy (Group

A) compared to patients who did not achieve a cure (Group B). Epithelial cells, T-lymphocytes, natural killer cells (NKs) and other immune cells release bioactive lipids, such as arachidonic acid, in response to microbial infections.¹⁵ Research indicates that HBV and hepatitis C virus (HCV) can be inactivated by alpha-linolenic acid (ALA), gamma-linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA).¹⁵ It is plausible, therefore, that gamma-linolenic acid can also play a role in deactivating HBV through the linoleic acid metabolism pathway.

Kaempferide, a phytochemical derived from the rhizomes of the ginger family plant *kaempferia*, has garnered attention due to its multiple benefits, including anticancer, cancer prevention, antiviral, antibacterial, antioxidant and anti-inflammatory properties.¹⁶ While the current study identified kaempferide as one of the three differential metabolites that were found enriched in Group A patients, its metabolic pathway has not been found in existing relevant metabolomics databases. Studies have reported that kaempferide has inhibitory effects on HBV, likely through interference with the virus's Pol/RT (polymerase/reverse transcriptase) and core proteins and by increasing the production of IFN-beta (β) and IFN-gamma (γ).¹⁷ In particular, Parvez MK et al.¹⁸ demonstrated that at a concentration of 10 μ g/ml, kaempferide effectively suppressed HBsAg and hepatitis B e-antigen (HBeAg) synthesis in HepG2.2.15 cells, underscoring its potential as a predictive biomarker for the clinical cure of chronic HBV infection.

Guanidoacetic acid ranked first among the differential metabolic products in Group A patients compared to those who did not achieve a cure (Group B), with $p=2.56 \times 10^{-8}$. Guanidoacetic acid can be synthesised in the kidneys, liver and pancreas, serving as a precursor to creatine, and is involved in the metabolic pathways of numerous amino acids, such as glycine, serine, threonine, arginine and proline. It has been reported¹⁴ that HBV replication can lead to the depletion of amino acids. It is plausible that the increased content of guanidinoacetic acid, as reported in Group A patients, may enhance the synthesis of creatine and amino acids to some extent. One of the indicators of the clinical cure of chronic HBV infection is the conversion of HBV DNA to negative. It is plausible that the increase in guanidine acetic acid observed in the cured patients (Group A) may be related to the normalisation of amino acid and creatine metabolism after HBV reversal. To the best of our knowledge, the current study is the first to report on guanidine acetic acid in the metabolomics of clinically cured hepatitis B patients. Furthermore, chronic HBV infection disrupts the oxidative-antioxidant balance,

and interferon alpha-2b (IFN α -2b) has been shown to improve this balance, particularly in treatment-responsive patients.¹⁹ As guanidoacetic acid can act as both a superoxide and an antioxidant compound, affecting the oxidative-antioxidative system, the high level of guanidoacetic acid in Group A patients who achieved a cure further supports the value of this metabolite as a potential biomarker.

Uracil 5-carboxylate ranked second among differential metabolites identified in this study ($p=5.94E-05$). Its related metabolic pathways could not be found in the metabolomics databases, indicating that this metabolite could provide a feasible direction for subsequent research.

Caffeine, as a chemical inhibitor of the ataxia-telangiectasia mutated (ATM)- and Rad3-related (ATR) protein in HBV-infected cells, can reduce HBV DNA levels by 70%.²⁰ Treating HBV-infected cells with caffeine inhibits the effects of HBV infection-induced upregulation of major histocompatibility complex class I-related protein A (MICA), thereby inhibiting liver damage caused by HBV infection.²¹ It is plausible, therefore, that caffeine is positively associated with HBV inhibition and reduction of liver damage. Further research is needed to explore the molecular mechanisms and the impact of caffeine on the clinical cure rates in individuals with chronic HBV infection.

3-Ketosphingosine was among the top-ranked differential metabolites and showed lower abundance in Group A than in Group E ($FC=0.90$). Sphingosine belongs to the class of sphingolipids (SLs) and is a component of the cell membrane. SLs have been implicated as regulators of the HBV life-cycle and are associated with virological progression.²² However, the value of SL as a potential new predictive factor for the course of HBeAg-negative HBV is still unclear. Zhang L et al.²³ found that peretinoin, a non-cyclic retinoid, inhibits HBV replication by inhibiting the sphingosine metabolic pathway in vitro. Therefore, the altered level of 3-ketosphingosine may reflect changes in sphingolipid metabolism associated with clinical cure; however, its specific role requires further investigation.

The current study is also the first to identify two differential metabolites—L-2-hydroxyglutaric acid (L-2HG) and 3,4-dihydroxybenzeneacetic acid—that were differentially altered in Group A compared with Group W. L-2HG was decreased, whereas 3,4-dihydroxybenzeneacetic acid was increased. L-2HG exists in two enantiomeric forms, D- and L-2HG, both of which are associated with tumour progression through impacts on energy metabolism and epigenetic regulation.²⁴ The 3,4-dihydroxybenzeneacetic acid has previously been mainly studied in the context of neurodegenerative diseases²⁵ and its role in HBV infection

remains unclear. The altered levels of these metabolites suggest their potential as biomarkers for disease progression or treatment response in chronic HBV infection, though their specific mechanisms and clinical significance require further investigation.

The current study has limitations. The sample size for this preliminary, exploratory metabolomics study was not determined by a formal power calculation prior to patient enrolment, which may affect the statistical power to detect smaller but potentially significant metabolic differences. The sample size was primarily determined based on the availability of eligible patients with chronic HBV infection who achieved clinical cure under the specified drug interventions within a single centre for a fixed duration. Furthermore, the mechanistic interpretations proposed for individual metabolites were derived largely by extrapolation from studies conducted in other disease contexts or in vitro/animal models, as direct functional data linking these metabolites to HBV clinical cure are not yet available; these interpretations should therefore be regarded as hypothesis-generating rather than confirmatory, pending targeted validation. Despite the limitation, the current study provides valuable initial insights into the metabolomic profiles associated with HBV clinical cure, which warrants further validation in larger, prospective studies.

Conclusion

A total of 52 metabolites were identified that are significantly different in patients who were clinically cured after treatment with ETV in combination with PGE-IFN α compared to those treated with ETV alone or left untreated. Of these, 9,10-DHOME, 12,13-DHOME and kaempferide were found to be involved in 42 metabolic pathways, including linoleic acid metabolism, caffeine metabolism and amino acid metabolism. These metabolites may have important implications for the clinical cure of chronic HBV infection. Additionally, metabolites such as guanidinoacetic acid, caffeine and 5-carboxy uracil may potentially contribute to clinical cure.

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Author Contribution:

PP, HX, XK, JW & WZ: Concept, design, data acquisition, analysis, interpretation, drafting, revision, final approval and agreement to be accountable for all aspects of the work.