Circulating metabolites are associated with persistent elevations of ALT in patients with chronic hepatitis B with complete viral suppression

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Abstract

Background & Aims: Hepatitis B virus (HBV) can be completely suppressed after antiviral treatment; however, some patients with chronic hepatitis B (CHB) still exhibit elevated alanine aminotransferase (ALT) levels and sustained disease progression. The aim of this study was to provide novel insights into the mechanism and potential predictive biomarkers of persistently elevated ALT (PeALT) in patients with CHB after complete viral inhibition. **Methods**: CHB Patients with undetectable HBV DNA at least 12 months after antiviral treatment were enrolled from a prospective, observational cohort. Correlations between plasma metabolites and the risk of elevated ALT were examined using multivariate logistic regression. **Results**: Of the 1238 patients with CHB who achieved complete viral suppression, 40 (3.23%) had PeALT levels during follow-up (median follow-up: 2.42 years). Additionally, 40 patients with persistently normal ALT (PnALT) levels were matched 1:1 as controls. Ser-Phe-Ala (variable importance in projection [VIP] = 4.28), Lys-Ala-Leu-Glu (VIP = 4.49), 3-methylhippuric acid (VIP = 3.04), 3-methylxanthine (VIP = 2.62), and 7-methylxanthine (VIP = 3.35) were identified as critical differential metabolites between the two groups and independently associated with PeALT risk. Ser-Phe-Ala and Lys-Ala-Leu-Glu levels could be used to discriminate patients with PeALT from those with PnALT. Furthermore, *N*-acetyl-l-methionine (NALM) demonstrated the strongest negative correlation with ALT levels. NALM supplementation alleviated liver injury and hepatic necrosis induced by carbon tetrachloride in mice. **Conclusions**: Changes in circulating metabolites may contribute to PeALT levels in patients with CHB who have achieved complete viral suppression after antiviral treatment.

Circulating metabolites are associated with persistent elevations of ALT in patients with chronic hepatitis B with complete viral suppression

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Abbreviations

HBV, hepatitis B virus; ALT, alanine aminotransferase; CHB, chronic hepatitis B; IL, interleukin; PeALT, persistently elevated ALT; CAP, controlled attenuation parameter; LSM, liver stiffness measurement; PnALT, persistently normal ALT; OPLS-DA, orthogonal projections to latent structures discriminant analysis; VIP, variable importance in projection; KEGG, Kyoto Encyclopedia of Genes and Genomes; PBS, phosphate buffered saline; ROC, receiver operating characteristic; AUC, area under the ROC curve; BMI, body mass index; TG, triglyceride; NALM,*N* -acetyl-l-methionine; 11-DHC, 11-dehydrocorticosterone; 3-Mx, 3-methylxanthine; 7-Mx, 7-methylxanthine; 3-MA, 3-methylhippuric acid

Ethics and Integrity Policies Statements

Conflict of interest disclosure

All authors declare that they do not have conflict of interests relevant to this manuscript.

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Data availability statement

The data that support the findings of this study are available from the authors, upon reasonable request.

Ethics approval statement

All of the survey protocols were approved by the Ethics Committee of Nanfang Hospital (Guangzhou, China).

Patient consent statement

Written informed consent was obtained from all of the participants.

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Not applicable

Clinical trial registration

Not applicable

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levels and sustained disease progression. The aim of this study was to provide novel insights into the mechanism and potential predictive biomarkers of persistently elevated ALT (PeALT) in patients with CHB after complete viral inhibition.

Methods : CHB Patients with undetectable HBV DNA at least 12 months after antiviral treatment were enrolled from a prospective, observational cohort. Correlations between plasma metabolites and the risk of elevated ALT were examined using multivariate logistic regression.

Results : Of the 1238 patients with CHB who achieved complete viral suppression, 40 (3.23%) had PeALT levels during follow-up (median follow-up: 2.42 years). Additionally, 40 patients with persistently normal ALT (PnALT) levels were matched 1:1 as controls. Ser-Phe-Ala (variable importance in projection [VIP] = 4.28), Lys-Ala-Leu-Glu (VIP = 4.49), 3-methylhippuric acid (VIP = 3.04), 3-methylxanthine (VIP = 2.62), and 7-methylxanthine (VIP = 3.35) were identified as critical differential metabolites between the two groups and independently associated with PeALT risk. Ser-Phe-Ala and Lys-Ala-Leu-Glu levels could be used to discriminate patients with PeALT from those with PnALT. Furthermore, *N* -acetyl-l-methionine (NALM) demonstrated the strongest negative correlation with ALT levels. NALM supplementation alleviated liver injury and hepatic necrosis induced by carbon tetrachloride in mice.

Conclusions : Changes in circulating metabolites may contribute to PeALT levels in patients with CHB who have achieved complete viral suppression after antiviral treatment.

Abstract word count: 244

Keywords: chronic hepatitis B; alanine aminotransferase; metabolites; *N* -acetyl-l-methionine

Lay summary

1. Alterations in the levels of circulating metabolites specific to patients with chronic hepatitis B (CHB) exhibiting persistently elevated alanine aminotransferase (PeALT) levels were identified.
2. ALT levels were most strongly negatively correlated with levels of*N* -acetyl-l-methionine (NALM); Supplementation with NALM alleviated hepatocyte necrosis in a CCl_4-induced mouse model of acute liver injury.
3. The identified key differential metabolites may serve as potential biomarkers and/or therapeutic targets for PeALT in patients with CHB.

Introduction

Hepatitis B virus (HBV) infection is a global public health concern, affecting approximately 300 million people and resulting in over 300,000 deaths every year due to cirrhosis, liver failure, or hepatocellular carcinoma.[1,2] Antiviral treatment with nucleoside analogues can effectively inhibit HBV replication and delay disease progression.[3,4] Several pivotal registration studies have shown that after five years of antiviral treatment with entecavir or tenofovir, 94%–99% of patients achieved undetectable HBV DNA and 80%–87% of patients achieved normalization of alanine aminotransferase (ALT),[5-7] with approximately 13%–20% of patients still showing elevated ALT even after long-term viral suppression.[8] Our previous study also reported that 34.8% of patients with chronic hepatitis B (CHB) had complete viral suppression and increased ALT during a follow-up of 18.36 months.[9] Multiple studies have reported that elevated ALT during antiviral therapy is associated with an increased risk of liver events, cirrhosis, and hepatocellular cancer in patients with CHB.[10-13] Moreover, an increase in ALT inhibits the accurate assessment of the disease stage and progression of CHB.[14] However, the reason and mechanism underlying elevated ALT in patients with CHB after viral suppression have not been fully explored.

Various studies have explored the reasons for elevated ALT during antiviral therapy. An intermittent increase in ALT during antiviral therapy is generally considered an immune response to HBV infection, indicating changes in CHB disease activity.[15]Additionally, persistent ALT elevation is typically associated with metabolic syndrome and liver steatosis.[16-18] However, the persistent elevation of ALT in patients with CHB cannot be explained completely by these factors. Numerous studies have recently found that metabolites play

a critical role in the development of liver inflammation.[19] For example, 3,4-dihydroxyphenylpropionic acid, a metabolite of gut microbiota, can suppress macrophage activation and improve hepatic ischemia/reperfusion injury via inhibiting histone deacetylase activity.[20]Additionally, the endogenous metabolite phosphonic acid can alleviate acetaminophen-induced liver injury by inducing the release of interleukin (IL-6) from adipose tissue.[21] A recent study showed that 2-oleoylglycerol produced by triacylglycerol metabolism promotes western diet-induced liver inflammation and fibrosis in mice through activating macrophages and hepatic stellate cells.[22] Overall, these findings imply that metabolites possibly play a role in ALT elevation in patients with CHB. Furthermore, alterations in circulating metabolites are associated with disease activity and progression in patients with CHB.[23-25] A study with 10 years follow-up found that dysregulation of methionine and branched-chain amino acid-related metabolites were associated with progression of CHB.[23]In addition, lipid and bile acid-related metabolites are associated with liver inflammation and fibrosis in patients with CHB. However, the association between elevated ALT and circulating metabolites in patients with CHB remains unclear.

In this study, we investigated the association between the levels of circulating metabolites and the risk of persistently elevated ALT (PeALT) in patients with CHB with complete viral suppression. Additionally, the potential role of differential metabolites in liver injury was primarily explored using animal models. Our aim was to provide novel insights into the mechanism and potential predictive biomarkers of PeALT in patients with CHB after complete HBV inhibition.

Methods

Study participants

This study was nested in a real-life prospective observational CHB cohort (Search-B cohort: NCT02167503). The study design and inclusion criteria for the Search-B cohort have been previously described.[26] Patients with CHB who achieved undetectable HBV DNA levels for at least 12 months after antiviral treatment at Nanfang Hospital between September 2015 and July 2017 were enrolled in this study. Patients meeting any of the following exclusion criteria were excluded: (1) coinfection with hepatitis C virus and hepatitis D virus; (2) diagnosis of malignant tumor; (3) excessive alcohol consumption (>21 units of alcohol per week for men and >14 units of alcohol per week for women) in the previous six months; (4) receiving medications with known effects on ALT, such as Chinese traditional medicines, anti-tuberculosis drugs, lipid-lowering drugs, nonsteroidal anti-inflammatory drugs, and antibiotics; (5) diagnosis of systemic inflammatory diseases, diabetes, and autoimmune liver disease; (6) HBV DNA [?] 20 IU/mL during follow-up; and (6) loss of follow-up.

Written informed consent for clinical data and blood sample collection was obtained from each patient during recruitment. The study protocol was approved by the Ethics Committee of Nanfang Hospital (Guangzhou, China).

Data collection

All subjects were followed up every six months. Baseline clinical and laboratory data were obtained through physical examination, questionnaire, routine blood test, FibroScan (controlled attenuation parameter [CAP] and liver stiffness measurement [LSM]), and virological examination. The participants were required to fast for at least 10 h before blood collection. Plasma and serum were collected and stored within 2 hours at -80 °C. Serum ALT levels were measured using an automatic biochemical analyzer (Olympus, Tokyo, Japan) according to the standard protocol. HBV DNA levels and serological markers were measured using the Roche COBAS TaqMan platform (lower limit of detection for HBV DNA: 20 IU/mL) and the Elecsys immunoassay (Roche, Basel, Switzerland) at the Hepatology Unit of Nanfang Hospital.

Definitions

Complete viral suppression was defined as HBV DNA levels below the detection limit or undetectable for more than 12 months. ALT levels exceeding 35 U/mL in males and 25 U/mL in females were defined as ALT

elevation.[14] In this study, the serum ALT elevation at all visits was defined as PeALT. If the ALT level was normal at all visits, it was defined as persistently normal ALT (PnALT).

Widely targeted metabolomics analysis

Widely targeted metabolomics was performed as previously described.[27,28] Briefly, 50 μL of plasma was transferred to an extraction solution (acetonitrile:methanol = 1:4, v/v) containing internal standards and then centrifuged at 12,000 rpm for 10 min (4 °C). The supernatant (180 μL) was collected for further analysis. Metabolites were separated and identified using ultra performance liquid chromatography (ExionLC AD, https://sciex.com.cn/), quadrupole-time of flight (TripleTOF 6600, AB SCIEX), and triple quadrupole-linear ion trap mass spectrometry (QTRAP®, *https://sciex.com/*). The orthogonal projections to latent structures discriminant analysis (OPLS-DA) model and variable importance in projection (VIP) values were generated using the R package MetaboAnalystR. Metabolites with p < 0.05, VIP [?] 1, and fold change [?] 1.2 or [?] 0.83 were considered significantly differential metabolites. Pathway enrichment analyses for these metabolites were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (*http://www.genome.jp/kegg*).

Animals and carbon tetrachloride (CCl_4)-induced liver injury

Eight-week-old C57BL/6 mice were purchased from SPF Biotechnology Co., Ltd. (Beijing, China) and bred in a specific pathogen-free facility at the Experimental Animal Center of Nanfang Hospital. Food and water were provided ad libitum under a 12/12 h light/dark cycle.

To induce acute liver injury, mice were administered one intraperitoneal injection of 10 mL/kg of 5% CCl_4 (Rhawn Co., Ltd., Shanghai, China) dissolved in corn oil, and the control group was administered one intraperitoneal injection of just corn oil (Macklin, Shanghai, China). Mice were treated with metabolites or phosphate buffered saline (PBS) at 2 h after injection of CCl_4. Several available differential metabolites were divided into water-soluble and non-water-soluble metabolites. For the non-water-soluble metabolites, mice with liver injury were administered 100 mg/kg of the corresponding metabolites by oral gavage. For water-soluble metabolites, mice with liver injury were intraperitoneally injected with 100 mg/kg of the corresponding metabolites. Blood was collected 24 and 48 h after CCl_4 injection. All mice were sacrificed 72 h after injection, and blood and liver samples were collected. The protocols for animal experiments were approved by the Institutional Animal Ethics Committee of the Experimental Animal Center of Nanfang Hospital.

Histological analysis

The liver tissue of mice was fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned into 4 μm-thick slices. For histological analysis, the sections were stained with hematoxylin and eosin.

Statistical analysis

Data analysis was performed using SPSS (version 20.0; IBM Corp., Armonk, NY, USA). The distribution of data was checked using the Shapiro–Wilk test. Continuous data with a normal distribution were expressed as means ± standard deviation; otherwise, data were expressed as medians with interquartile ranges. Categorical data are presented as counts (%). Differences between groups were examined using the Student's t-test, Mann–Whitney U test, and chi-square test. Correlations between the differential metabolites and ALT levels were analyzed using Spearman's correlation coefficient. Multivariate logistic regression was used to assess the association between metabolites and the risk of PeALT. The ability to distinguish between patients with PeALT and PnALT using different metabolites was evaluated using the area under the receiver operating characteristic (ROC) curve (AUC). The level of statistical significance was set at $p < 0.05$ (two-sided).

Results

Demographic and clinical information of participants

As shown in Figure 1A, 1709 patients with CHB who had undetectable serum HBV DNA levels for at least

12 months after antiviral treatment were screened. In total, 208 participants were excluded at baseline, and 263 participants were excluded during follow-up (median follow-up: 2.42 years). Finally, 1238 patients with CHB were included in the analysis. Among these patients, 510 (41.19%) experienced elevated ALT during follow-up, 40 (3.23%) showed PeALT at all visits, and only 55.57% maintained PnALT.

To further investigate the potential reason for elevated ALT, we matched 40 patients with PeALT and 40 with PnALT using propensity score matching. Confounding factors, including sex, age, body mass index (BMI), follow-up duration, serum triglyceride (TG) and cholesterol levels, and CAP, were well-matched between these two groups. Baseline demographics and clinical information of the patients are summarized in Table 1. The serum ALT (49.5 [42, 64.5] vs. 21 [14, 25.75)]) and AST (31 [25.25, 38] vs. 20 [19, 25] levels were significantly higher in the PeALT group than in the PnALT group. The dynamic changes in ALT and AST levels are shown in Figure 1B and 1C. In addition to ALT levels, the serum AST level in patients with PeALT was consistently and significantly higher than that in patients with PnALT, indicating sustained liver injury.

Significant differences in circulating metabolites between the PeALT and PnALT groups

To investigate the role of circulating metabolites in PeALT, plasma metabolomics was conducted, and 1051 metabolites were detected. Greater than 60% of the metabolites identified in the plasma belonged to the following six categories: amino acids and their metabolites (20.85%), benzene and its substituted derivatives (12.61%), organic acids and their derivatives (11.75%), heterocyclic compounds (10.62%), fatty acids (7.49%) and alcohol and amines (5.69%) (Figure 2A). OPLS-DA showed a clear separation in the metabolite profiles of the PeALT and PnALT groups (R2Y = 0.966, Q2 = 0.509, Figure 2B). The expression of 17 metabolites was significantly upregulated, whereas the expression of 23 metabolites was significantly downregulated in the PeALT group compared to that in the PnALT group (Figure 2C). KEGG pathway analysis revealed that the mTOR signaling pathway, drug metabolism-cytochrome P_{450}, amino acid biosynthesis, D-amino acid metabolism, and central carbon metabolism in cancer were primarily enriched for the differential metabolites (Figure 2D). For further analysis, we focused on the top ten differential metabolites ranked by VIP, which reflected the level of influence of the metabolites. Levels of Lys-Ala-Leu-Glu (VIP = 4.49), 11-dehydrocorticosterone (VIP = 4.17), L-beta-phenylalanine (VIP = 3.65), *N* -acetyl-l-methionine (NALM) (VIP = 3.28), 3-methylxanthine (3-Mx) (VIP = 2.62), 7-methylxanthine (7-Mx) (VIP = 2.35), and Pro-His (VIP = 2.32) were significantly lower, while levels of Ser-Phe-Ala (VIP = 4.28), 3-methylhippuric acid (3-MA) (VIP = 3.04), and α-ketoglutaric acid (VIP = 2.82) were significantly higher in patients with PeALT than in patients with PnALT (Figure 2E and 2F).

Differential plasma metabolites are associated with PeALT risk

We evaluated the correlations between the levels of differential metabolites and clinical parameters. As shown in Figure 3A and 3B, Ser-Phe-Ala and NALM had the strongest negative and positive correlation with ALT and AST levels, respectively, implying that these two metabolites may be associated with liver injury in patients with CHB. Moreover, 3-Mx was negatively associated with white blood cells and neutrophils. Multivariate logistic regression analysis further confirmed that these metabolites were associated with PeALT risk, even after adjusting for age, sex, BMI, low density lipoprotein, platelet, total cholesterol, and TG (Table 2). These findings suggest that the correlation between serum metabolites and the risk of elevated ALT levels may be independent of BMI and metabolic syndrome. The ROC curve revealed that Ser-Phe-Ala (AUC = 0.843, 95% CI 0.75–0.93) and Lys-Ala-Leu-Glu (AUC = 0.839, 95% CI 0.744–0.934) can be used to distinguish patients with PeALT from those with PnALT (Figure 3C). The findings indicate that plasma metabolites are associated with PeALT risk and may be potential biomarkers for diagnosing PeALT.

NALM improves CCl_4-induced liver injury and may be a potential treatment option for PeALT in patients with CHB

To explore the potential role of differential metabolites in ALT elevation, we established a mouse model of liver injury by inducing liver damage using CCl_4 to simulate the ALT elevation observed in patients with CHB (Figure 4A). After 24 h of CCl_4 stimulation, the serum ALT levels of mice significantly increased, peaking

at 48 h, and then decreasing significantly at 72 h, indicating regression in liver injury. Notably, we found that at 48 and 72 h after CCl_4 stimulation, ALT levels in the CCl_4+NALM (i.p.) group were significantly lower than those in the CCl_4+PBS (i.p.) group, indicating that NALM supplementation alleviated the liver injury induced by CCl_4 (Figure 4B). In addition, ALT levels in the CCl_4+3-Mx (i.g.) group were significantly reduced compared to those in the CCl_4+PBS (i.g.) group at 48 h. At 72 h, the ALT levels in the CCL_4+3-MA (i.g.) group were significantly higher than those in the CCl_4+PBS (i.g.) group, indicating that 3-MA may hinder the regression of liver injury (Figure 4C). Subsequently, we evaluated the roles of these metabolites on AST levels. NALM, 7-Mx and 3-Mx significantly inhibited the increase in AST caused by CCL_4 (Figure 4D and 4E). Further pathological analysis suggested that NALM supplementation significantly reduced CCl_4-induced necrosis compared to that in the CCl_4+PBS (i.p.) group (Figure 4F and 4G). Overall, NALM may improve liver injury, promote the recovery of liver function, and be a potential treatment for PeALT in patients with CHB.

Discussion

In this study, we revealed for the first time an association between the levels of circulating metabolites and the risk of PeALT in patients with CHB who have achieved complete HBV suppression. Additionally, supplementation with NALM was observed to improve liver injury caused by CCl_4.

ALT is predominantly found in the liver cytoplasm, and its main physiological function is to catalyze the transformation of glutamic acid to alanine through transamination. Generally, 1% of damaged hepatocytes can enhance circulating ALT activity by at least one-fold. As a result, ALT levels are considered one of the most sensitive indicators of liver injury.[29] Our results indicate that 3.23% of patients with CHB who have achieved HBV suppression still experience PeALT after excluding factors such as excessive alcohol consumption, medication, and HBV activity. Previous studies reported that even mild elevation of ALT is associated with an increased risk of liver complications and more severe histological inflammation in patients with CHB.[30,31] Moreover, PeALT levels during antiviral therapy are independently associated with an increased risk of hepatocellular carcinoma in patients with CHB.[11]Therefore, clarifying the causes of elevated ALT in CHB patients has clinical significance.

Given the hub role of metabolites in the progression of liver disease, we investigated the effect of circulating metabolites on PeALT levels in patients with CHB. Metabolomic analyses indicated 17 upregulated and 23 downregulated metabolites in patients with CHB with PeALT compared to those in the PnALT group. Furthermore, the correlation between the identified differential metabolites and the risk of elevated ALT was evaluated. We found that even after adjusting for confounding factors, the top 10 differential metabolites were still significantly associated with the risk of elevated ALT, further indicating the critical role of circulating metabolites in PeALT.

Next, we investigated how plasma metabolites may cause an increase in ALT. Notably, many amino acid metabolism-related pathways, such as amino acid synthesis, D-amino acid metabolism, and arginine metabolism, were enriched for the differential metabolites. Multiple studies have shown that changes in circulating amino acids are associated with the progression of chronic liver disease. For example, citrulline, a metabolite produced by arginine metabolism, has been shown to improve non-alcoholic fatty liver in mice by protecting intestinal barrier function and preventing bacterial translocation to the liver.[32] By contrast, a decrease in glycine synthesis promoted western diet-induced liver injury and inflammation in mice by inhibiting glutathione production.[33] As a result, we assumed that plasma amino acids may be involved in the pathogenesis of PeALT. Further analysis revealed that levels of NALM, an amino acid derivative, showed the strongest negative correlation with both ALT and AST levels among the top 10 metabolites. This suggests that NALM plays a key role in the continuous increase in ALT levels. NALM is produced by the acetylation of methionine with acetic acid[34] and contributes to the clearance of reactive oxygen species and prevents oxidative stress *in vitro* and *in vivo*. [35,36] Our results indicate that NALM can significantly improve CCl_4-induced liver injury. Moreover, NALM can reduce CCl_4-induced hepatocyte necrosis, contributing to the repair of liver injury. These results imply that decreased NALM levels may affect the body's ability to repair liver damage, leading to a sustained elevation of ALT levels after acute inflammation.

In addition, circulating levels of 3-Mx and 7-Mx, derivatives of caffeine, were significantly lower in the PeALT group than in the PnALT group. Caffeine enters the liver after absorption in the gastrointestinal tract and is metabolized by cytochrome P450, producing a series of methylxanthine derivatives, including 3-Mx and 7-Mx.[37-39] These two metabolites directly participate in regulating the inflammatory response through adenosine receptors on the surface of immune cells.[40,41] Our results also showed a significant negative correlation between 3-Mx levels and the numbers of peripheral white blood cells and neutrophils. This suggests that 3-Mx may be involved in the negative regulation of the immune system. Moreover, we found that in the acute phase of liver injury, 3-Mx reduced the elevation of ALT and AST caused by CCl_4, indicating a potential protective effect of 3-Mx against liver injury. Another important differential metabolite was 3-MA, which is a metabolite of toluene and xylene; its levels are commonly used to monitor exposure to these environmental pollutants.[42,43] As a result, higher circulating levels of 3-MA in patients with PeALT than in those with PnALT may indicate greater exposure to toluene and xylene.[44]Exposure to these chemicals can lead to hepatocellular oxidative stress and liver damage in mice, which is possibly mediated by the metabolites of toluene and xylene.[45,46] Our results indicated that 3-MA can cause a delayed recovery in ALT levels in mice with acute liver injury, which may have led to a sustained elevation in ALT levels. Therefore, more clinical and basic research should be conducted to verify the correlation between PeALT and exposure to toluene, xylene, and their metabolites. Overall, these key differential metabolites, particularly NALM, may serve as potential therapeutic targets for PeALT in patients with CHB; however, further *in vitro* and *in vivo* experimental validation is necessary.

This study has several limitations. First, widely targeted metabolomics was used to analyze all plasma metabolites comprehensively and systematically. The relative concentrations of metabolites can be monitored; however, their absolute quantification remains a challenge. Hence, targeted metabolomics should be used to validate our results in the future. Moreover, owing to the short follow-up time and small sample size, we were unable to observe correlations between differential metabolites and disease progression in CHB patients with viral suppression. Another limitation of the present study is that we included CAP and LSM as regional substitutes for hepatic steatosis and liver fibrosis rather than liver biopsy, however, liver biopsy is not feasible in real-life large-sample prospective observational cohort studies.

To our knowledge, this is the first study to uncover the role of metabolites in liver inflammation in CHB patients with complete viral suppression, based on a long-term follow-up cohort and case-controlled studies. The present study revealed alterations in circulating metabolite levels in patients with CHB with PeALT. This may help to reveal the mechanism of ALT elevation in these patients and provide therapeutic targets.

Author contributions

DK Zheng, CH Cheng and YH Tang contributed to statistical analyses and manuscript writing. ZX Fang and YH Tang contributed to interpretation of the data. YC Chen, QH You, and XL Gao helped with collecting tissues from the mice. KF Wang, HQ Zhou, and ZX Lan provided advice and revised the manuscript. J Sun contributed to the study concept and design, critical revision of the manuscript, and administrative support. All of the authors critically reviewed and approved the final version of the manuscript.

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Table 1. Baseline characteristics of participants[+]

	PnALT[++]	**PeALT**	p**-value**[§]
Male, n [%]	32 [80]	33 [82.5]	0.775
Age, years	40.95 ± 10.78	40.82 ± 8.90	0.955
BMI, kg/m²	24.44 ± 3.55	24.49 ± 3.18	0.949
CAP, dB/m	221.42 ± 41.86	220.6 ± 38.80	0.927
TG, mmol/L	0.93 [0.65–1.41]	1.15 [0.7–1.61]	0.394
TC, mmol/L	4.26 [3.54–4.85]	4.64 [3.99–5.71]	0.092
Follow-up time, years	2.26 [1.97–3.44]	2.42 [1.67–3.48]	0.634
Liver cirrhosis, n (%)	17 [42.5]	14 [35]	0.491
HBeAg positive, n [%]	5 [12.5]	4 [10]	0.723
ALT, U/L	21 [14–25.75]	49.5 [42–64.5]	**0.0001**
AST, U/L	20 [19–25]	31 (25. 25–38)	**0.0001**
LSM, Kpa	8.15 [6.125–14.07]	7 [5.6–9.22]	0.172
ALB, g/L	44.6 [42.07–47.2]	45.6 [44–47.1]	0.368
AFP, ng/mL	3.29 ± 2.2	2.84 ± 1.53	0.288
GLU, mmol/L	5.2 [4.88–5.53]	5.07 [4.89–5.36]	0.600
HDL, mmol/L	1.04 [0.92–1.28]	1.08 [0.94–1.29]	0.507
LDL, mmol/L	2.69 ± 0.78	3.09 ± 0.73	**0.021**
WBC, 10^9/L	5.69 [4.99–6.68]	5.9 [5.19–5.9]	0.433
NEU, 10^9/L	3.29 [2.6–3.81]	3.32 [2.85–4.4]	0.433
PLT, 10^9/L	167.5 [105.2–210.2]	212 [134–257.2]	**0.011**

[+]Data are depicted as means ± standard deviation, median [interquartile range], or counts.

[++]Abbreviations: PnALT, persistently normal ALT; PeALT, persistently elevated ALT; BMI, body mass index; CAP, controlled attenuation parameter; TG, triglyceride; TC, total cholesterol; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; AST, aspartate transaminase; LSM, liver stiffness measurement; ALB, AFP, Alpha-fetoprotein; albumin; GLU, glucose; HDL, high density lipoprotein; LDL, low density lipoprotein; WBC, white blood cell; NEU, neutrophil; PLT, platelet.

[SS]p - values < 0.05 are bolded.

Table 2. Association between the levels of differential metabolites and the risk of persistently elevated alanine aminotransferase

Metabolites	**Unadjusted OR[++]**	**Unadjusted 95% CI (OR)**	**Unadjusted p-value**	**OR**	**Adjusted[+] 95% CI (OR)**	**p -value[§]**
Lys-Ala-Leu-Glu	0.014	0.002–0.101	**0.001**	0.001	0.001–0.027	**0.001**
Ser-Phe-Ala	5.423	2.505–11.743	**0.001**	5.53	2.425–12.626	**0.001**
11-Dehydrocorticosterone	0.127	0.048–0.337	**0.001**	0.076	0.021–0.268	**0.001**
l-beta-Phenylalanine	0.488	0.260–0.914	**0.025**	0.437	0.209–0.915	**0.028**
N-acetyl-l-methionine	0.018	0.002–0.161	**0.001**	0.015	0.001–0.169–	**0.001**
3-Methylhippuric acid	1.647	1.164–2.332	**0.001**	1.778	1.172–2.698	**0.007**
α-Ketoglutaric Acid	11.819	2.770–50.424	**0.001**	24.23	3.732–157.343	**0.001**
3-Methylxanthine	0.162	0.051–0.513	**0.002**	0.205	0.059–0.717	**0.013**
7-Methylxanthine	0.223	0.082–0.609	**0.003**	0.268	0.091–0.792	**0.017**
Pro-His	0.09	0.019–0.440	**0.003**	0.053	0.008–0.364	**0.003**

[+]Adjusted for age, gender, BMI, TC, TG, LDL, and PLT.

[++]Abbreviations: OR, odds ratio; CI, Confidence Interval.

[§]p -values < 0.05 are bolded.

[++]Abbreviations:

Figure legends

Figure 1. (A) Study flowchart. Dynamic changes in alanine aminotransferase (ALT) (B) and aspartate transaminase (AST) (C) levels during follow-up. * $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$. PeALT, persistently elevated ALT; PnALT, persistently nomal ALT.

Figure 2. Significant differences in circulating metabolites between the PeALT and PnALT groups. (A) Total number and main categories of identified metabolites. (B) OPLS-DA model and (C) Volcano plots of the PnALT vs. PeALT groups. (D) KEGG analysis results of enriched signaling pathways. (E) The top 10 differential metabolites were ranked by VIP values. (F) Relative intensity of the top 10 differential metabolites between the PeALT and PnALT groups. **$p < 0.01$; ***$p < 0.001$. OPLS-DA, orthogonal projections to latent structures discriminant analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; VIP, variable importance in projection.

Figure 3. Differential plasma metabolites associated with PeALT risk. (A) Correlations of the levels of differential metabolites with ALT levels, and (B) clinical parameters. (C) ROC curve for differential metabolites. *$p < 0.05$; **$p < 0.01$. ROC, receiver operating characteristic.

Figure 4. NALM improves carbon tetrachloride-induced liver injury. (A) Schematic diagram of the mouse experiment. (B, C) Comparison of serum ALT levels in each group at 24, 48, and 72 h. (D, E) Comparison of serum AST levels in each group at 72 h. (F, G) Representative images of hematoxylin and eosin staining and quantification of necrotic areas in the liver. Scale bar = 100 μm. *$p < 0.05$; **$p< 0.01$; ***$p < 0.001$. "ns" indicates not significant. NALM, N -acetyl-l-methionine; 11-DHC, 11-dehydrocorticosterone; 3-Mx, 3-methylxanthine; 7-Mx, 7-methylxanthine; 3-MA, 3-methylhippuric acid.