*Article*

# Moderate, Rather than Vigorous Exercise Improves Plasma Amino Acid Profiles in Patients with Nonalcoholic Fatty Liver Disease

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**Abstract:** Objective: This study was undertaken to investigate the relationship between amino acids (AAs) and nonalcoholic fatty liver disease (NAFLD), and how AAs changed following long-term exercise training under different exercise intensities. Methods: NAFLD participants (n = 220) were recruited and randomly assigned to control, moderate exercise and vigorous exercise groups with a 6-month follow-up. Clinical characteristics were carefully calculated and plasma AAs concentrations were determined using a validated ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method. Results: At baseline, AAs concentrations were closely associated with clinical characteristics in NAFLD, particularly, the sum of branched chain amino acids (BCAAs) positively associated with intrahepatic triglyceride (IHTG) content (r = 0.18, $p$ = 0.007). After 6-month exercise intervention, several AAs concentrations altered, and different exercise intensities exerted inverse effects on histidine, serine, glutamine, valine, tyrosine, and tryptophan concentrations changes, particularly, moderate exercise was much more efficient on decreasing BCAAs than vigorous exercise with a significant difference ($p$ = 0.0008). Conclusion: Several AAs was closely associated with IHTG content in NAFLD patients, and 6-month moderate exercise more strongly reduced AAs concentrations, particularly BCAAs, compared to vigorous exercise. These findings suggest that exercise intensity optimization could enhance the metabolic benefits of exercise therapy in NAFLD.

**Keywords:** nonalcoholic fatty liver disease; exercise intensity; amino acid

## 1. Introduction

Nonalcoholic fatty liver disease (NAFLD) has become a pandemic disease driven by advancements in people's living standards, which is the hepatic manifestation of the metabolic syndrome and insulin resistance [1]. Many studies have demonstrated that exercise is a recommended treatment due to its potential to decrease visceral adipose tissue, liver fat, body fat, weight, and the improvement of cardiovascular risk factors [2–4].

Recently, Targeted and non-targeted metabolomics studies have discovered that altered circulating levels of amino acids (AAs) were typical features in NAFLD subjects, metabolic homeostasis of which plays an important role in the development and progression of NAFLD [5–8]. In particular, branched chain amino acids (BCAAs, leucine, isoleucine, and valine) have been found to be elevated in NAFLD patients [9], and strongly associated with increased risk of NAFLD [10], insulin resistance, obesity [11,12], and future clinical decompensation in NAFLD [13]. It was also revealed that the changes in hepatic BCAA composition were strongly associated with transcriptomic metabolism profiles during the progression of NAFLD [14]. Various exercise intensities impacted plasma metabolic profile differently [15,16]. Babu AF et al. [5] investigated via non-targeted metabolomics analysis and found that high-intensity interval training increased the levels of amino acids and their derivatives, such as leucine, methionine,

and threonine, in adipose tissue and plasma, while decreasing related amino acids, such as glutamine, ornithine, and peptides in urine and stool. The results suggest that altered amino acid metabolism in adipose tissue may mediate the beneficial effects of exercise on NAFLD by promoting mitochondrial function and regulating glucose and lipid metabolism. Stone M et al. [17] detected amino acids in plasma and sweat, and found that the concentration of plasma amino acids decreased while excretion via sweat glands increased. This indicates that an increase in exercise intensity can enhance amino acid metabolism. Quiroga R et al. [10] revealed via metabolomics analysis that exercise reduces the levels of branched-chain amino acids (such as isoleucine and leucine) in the feces of obese children. Meanwhile, exercise can also affect the levels of metabolites like glutamate by modulating the gut microbiota, thereby influencing obesity-related metabolic risks. Other studies have shown that high-intensity interval exercise has no significant effect on the total amino acid concentrations in plasma and red blood cells, but individual amino acid analyses showed significant interaction effects for alanine and α-aminoadipic acid [18]. Additionally, previous studies have shown that Tai Chi exercise can downregulate the levels of homocysteine and methionine sulfoxide, upregulate amino acid metabolites such as L-fucose and pipecolic acid, and regulate amino acid metabolism and its associated pathways, thereby improving motor symptoms in Parkinson's disease patients [19]. To date, few studies have explored the changes in circulating AAs following exercise training [20–23], and the relationship between AAs metabolism and exercise intensity was little known. Our previous clinic trial demonstrated that intrahepatic triglyceride (IHTG) content in NAFLD patients were decreased significantly by both 6 month vigorous and moderate exercise training, and high intensity exercise offers no additional benefit to moderate intensity exercise in reducing liver fat [4,24], although vigorous exercise was more effective on weight, waist circumference, body fat and visceral fat. However, the metabolic profile of plasma AAs in these NAFLD subjects are unknown, and whether AAs concentrations are altered after different exercise intensity training is not well understood [25].

Thus, we performed a targeted metabolomics study on eighteen plasma AAs concentrations analysis in NAFLD subjects using a validated ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method, and analyzed the correlation between AAs concentrations and clinical characteristics, especially IHTG content, and also assessed the effect on AAs concentrations alteration after long term vigorous and moderate exercise training.

## 2. Methods

### *2.1. Study Participants*

Study participant and protocol were described in our previous study [26], briefly, a total of 220 individuals with NAFLD were selected and randomly assigned to control group (n = 74), moderate exercise group (n = 73, brisk walking 150 min per week at 45–55% of maximum heart rate), and vigorous exercise group (n = 73, jogging 150 min per week at 65–80% of maximum heart rate for 6 months). Participants in the control group were instructed not to change their physical activity routine. Participants assigned to the vigorous-moderate exercise group were required to participate in 5 vigorous exercise sessions each week, supervised by a study physician at a local community health center. Participants in the moderate exercise program were required to wear pedometers (Omron Healthcare) and record their daily exercise in a log, which was reviewed weekly by study staff. Participants received follow-up telephone calls from study staff twice per week to assess their adherence to the program and provide suggestions for improvement. Before starting the vigorous and moderate exercise programs, participants were trained for 2 to 4 weeks to achieve the appropriate exercise intensity.

Blood samples were collected in the early morning after an overnight fast, and then the plasma samples were stored at −80 °C until analysis. In this present study, clinical characteristics and plasma AAs concentrations of all study participants at baseline and 6-month exercise intervention were studied. The current study was approved by the human ethics committees of the First Affiliated Hospital of Xiamen University. The study was conducted following the Declaration of Helsinki guidelines. This protocol was also registered at ClinicalTrials.gov as NCT01418027. Written informed consent was obtained from all individual participants included in the study.

### *2.2. Determination of Plasma AAs*

Plasma AAs concentrations were determined accurately using Agilent 6460 triple stage quadrupole mass spectrometer equipped with an Agilent 1290 HPLC system (Agilent Technologies, Santa Clara, CA, USA), after sample preparation, chromatographic conditions and mass spectrometer parameters for AAs analysis were carefully optimized, and linearity, sensitivity, intra-day and inter-day precision, accuracy, and matrix effects were strictly performed for method validation (data were provided in Supplementary Materials).

20 μL of plasma was added 1 μL L-phenyl-d5-alanine (10 μg/mL) and 80 μL acetonitrile, the mixture was vortex-mixed for 30 s and centrifuged at 19,000× *g* for 10 min at 4 °C, 40 μL of the supernatant was then mixed with 120 μL water, then the aliquot of which (5 μL) was injected into the UHPLC-MS/MS system for analysis (LC-MS condition for determination of eighteen AAs were provided in Supplementary Materials). Data of AAs concentrations were acquired and processed using Agilent Mass Hunter Workstation Data Acquisition and Quantitative Analysis B.07.00 (Agilent Technologies, Santa Clara, CA, USA).

Using this approach, we were able to quantify eighteen AAs concentrations in human plasma: taurine (Tau), methionine (Met), glutamine (Gln), histidine (His), lysine (Lys), leucine (Leu), isoleucine (Ile), valine (Val), arginine (Arg), phenylalanine (Phe), tryptophan (Trp), proline (Pro), threonine (Thr), alanine (Ala), serine (Ser), glutamine (Gln), glycine (Gly), and tyrosine (Tyr).

### *2.3. Statistical Analysis*

Data were summarized using frequencies and counts for categorical variables and means and standard deviations for continuous variables. Data were analyzed according to participants' randomization assignments, regardless of their subsequent status. The general linear models were performed to assess the effects of exercise programs on the change in plasma AAs levels, with adjustment for age, sex, BMI at baseline, and value for the respective outcome traits at baseline. Pearson's correlation coefficient (for normally distributed variables) and Spearman's rank correlation (for non-normally distributed variables) were used for correlation analysis of plasma AAs levels and clinical characteristics at baseline. Heatmap analysis was performed using GraphPad Prism 7.0. SAS statistical software, version 9.4 (SAS Institute Inc), was used to obtain point estimates and SEs of the treatment effects and to test for differences between treatments. $p < 0.008$ (Bonferroni-adjusted $\alpha = 0.05/6$) was considered statistically significant.

## 3. Results

### *3.1. Clinical Characteristics of Study Participants*

A total of 220 eligible trial participants were recruited and followed up for 12 months [4]. In this study, only the 6-month intervention group was analyzed; 74 participants completed the 6-month intervention in the control group, 69 participants completed the 6-month moderate exercise and 68 participants completed the 6-month vigorous exercise.

Baseline clinical characteristics were balanced among the three groups (Supplement Table S4), including the general information and clinical parameters. After 6 month exercise training, IHTG content was reduced by both exercise intensities (5.0% reduction by vigorous exercise, $p < 0.001$; 4.2% reduction by moderate exercise, $p < 0.001$) with an equal effect ($p = 0.45$), while weight, waist circumference, body fat, visceral fat and blood pressure were only decreased significantly after vigorous exercise (Supplement Table S5).

### *3.2. Association between AAs Concentrations and Clinical Characteristics at Baseline*

Baseline plasma AAs concentrations of NAFLD subjects in control, moderate exercise, and vigorous exercise groups were summarized in Table 1. At baseline, there were no significant differences between these three groups on seventeen AAs concentrations, while plasma Phe concentrations were much higher in the moderate exercise group than the control ($p = 0.0012$) and the vigorous exercise group ($p = 0.0684$), respectively.

The type of AAs that was most closely associated with the main clinical characteristics of patients with NAFLD was determined using bivariate correlations. Most AAs showed a strong relationship with clinical parameters at baseline Figure 1; in particular, all kinds of BCAAs Ile, Val and Leu were positively associated with IHTG content, liver enzymes (alanine transaminase and glutamyl transpeptidase), weight, BMI (body mass index), waist circumference and visceral fat, and inversely associated with body fat and HDL-C, respectively. Furthermore, the correlation analysis between the sum of BCAAs and IHTG content was performed ($r = 0.18$, $p = 0.007$, Figure 2). Similarly, both aromatic AAs Phe and Tyr were positively correlated with weight, BMI, and waist circumference.

**Table 1.** Plasma concentration of AAs (μg/mL) of study participants at baseline.

| AAs | Control (n = 74) | Moderate Exercise (n = 73) | Vigorous Exercise (n = 73) | *p* Values |
|---|---|---|---|---|
| Orn | 6.1 (2.5) | 5.7 (1.9) | 6.3 (3.1) | 0.7183 |
| Lys | 7.8 (2.1) | 8.0 (1.8) | 8.7 (2.6) | 0.0460 |
| Arg | 5.8 (2.3) | 6.3 (1.8) | 6.0 (1.5) | 0.3621 |
| His | 10.6(1.5) | 10.6 (1.4) | 11.4 (4.0) | 0.1095 |
| Gly | 16.0 (3.5) | 16.3 (4.0) | 16.3 (6.9) | 0.7110 |
| Ser | 11.9 (1.8) | 12.1 (2.1) | 12.7 (2.9) | 0.6992 |
| Gln | 72.7 (11.1) | 72.1 (10.4) | 76.4 (7.9) | 0.0149 |
| Ala | 35.3 (6.3) | 36.2 (6.2) | 35.6 (5.7) | 0.7080 |
| Tau | 16.9 (3.4) | 16.2 (3.7) | 16.3 (4.0) | 0.4731 |
| Thr | 13.1 (1.9) | 12.8 (2.1) | 13.0 (2.9) | 0.6840 |
| Pro | 17.7 (4.6) | 18.6 (4.9) | 18.9 (4.8) | 0.2776 |
| Val | 22.4(4.0) | 23.3 (4.1) | 22.7(4.5) | 0.4155 |
| Met | 2.8 (0.6) | 2.8 (0.4) | 2.7 (0.5) | 0.9176 |
| Ile | 7.0 (1.7) | 7.2 (1.5) | 7.2 (1.8) | 0.6029 |
| Tyr | 9.0 (2.3) | 9.2 (1.7) | 9.1 (2.3) | 0.8630 |
| Leu | 15.1 (3.0) | 15.7 (2.9) | 15.2 (3.1) | 0.3535 |
| Phe | 11.8(2.0) | 12.4 (1.6) | 11.4 (2.0) | 0.0050 |
| Trp | 12.2 (2.0) | 12.5 (2.1) | 11.7 (2.1) | 0.9077 |

Data were presented as mean (standard deviation). $p < 0.008$ (0.05/6 comparisons) was considered statistically significant.

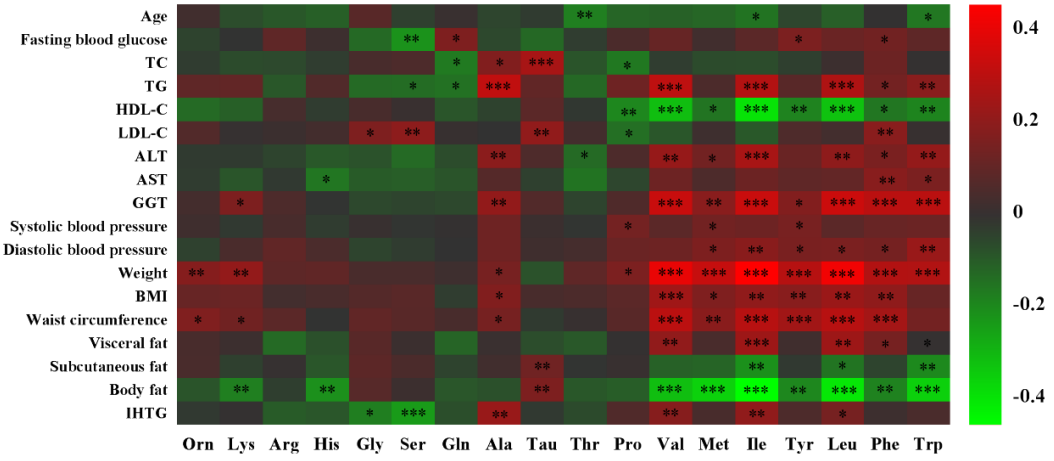


**Figure 1.** Heatmap analysis of correlation between AAs concentrations and clinical characteristics in NAFLD participants at baseline (n = 220). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

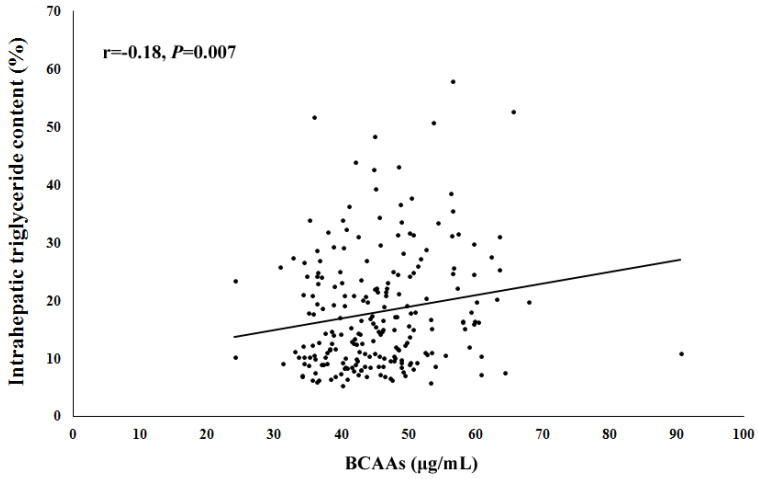


**Figure 2.** Correlation between the sum of BCAAs concentrations and IHTG content in NAFLD participants at baseline (n = 220).

### *3.3. Changes in Plasma AAs Concentrations Response to 6-Month Exercise Training*

As shown in Table 2, the concentrations of Orn, Lys, Arg, Thr, and Pro were not altered following the 6-month exercise training program. Compared to control, concentrations of His, Gly, Ser, Ala, Tau, Val, Ile, Tyr, Leu, Phe and Trp were decreased and concentrations of Gln was increased significantly after 6 month moderate exercise; concentrations of Gln was decreased and concentrations of His, Ser, Val, Met, Tyr and Trp was increased significantly after 6 month vigorous exercise, respectively. In addition, there were significant differences in the changes of Gly, Gln, Tau, Met, Ile, Leu, and Phe between moderate and vigorous exercise interventions. Furthermore, the changes in the sum BCAAs concentrations after 6-month intervention (Figure 3) were calculated; it was found that compared to vigorous exercise, moderate exercise ($p$ = 0.006) was much more efficient in decreasing BCAAs concentrations with a significant difference between these two exercise intensities ($p$ = 0.0008).

**Table 2.** Changes in plasma AAs concentrations after 6-month exercise intervention.

| AAs | Changes (95% CI) | | | *p* Values | | |
|---|---|---|---|---|---|---|
| | Control | Moderate Exercise | Vigorous Exercise | Moderate vs. Control | Vigorous vs. Control | Vigorous vs. Moderate |
| Orn | 0.6 (−0.6 to 1.8) | −0.1 (−1.0 to 1.6) | 0.6 (−0.2 to 2.1) | 0.9063 | 0.9247 | 0.9824 |
| Lys | 0.9 (−0.8 to 2.1) | 0.7 (−0.7 to 2.0) | 0.5 (−0.6 to 1.58) | 0.7241 | 0.4779 | 0.2946 |
| Arg | 0.5 (−1.2 to 1.2) | 0.7 (−0.7 to 1.9) | 0.4 (−0.40 to −0.8) | 0.0233 | 0.9253 | 0.0203 |
| His | 0.5 (−0.1 to 1.1) | 0.05 (−1.1 to 0.7) | 0.7 (0.03 to 1.8) | <0.0001 | 0.0002 | 0.1835 |
| Gly | 1.3 (−0.7 to 3.0) | −0.3 (−1.4 to 1.4) | 3.3 (1.5 to 5.1) | 0.0008 | 0.9771 | 0.0012 |
| Ser | 1.3 (0.4 to 2.7) | 0.3 (−0.8 to 1.1) | 2.2 (1.4 to 3.7) | <0.0001 | 0.0001 | 0.1633 |
| Gln | −4.8 (−9.9 to 2.1) | 0.5 (−4.4 to 7.4) | −5.0 (−8.0 to −2.0) | 0.0037 | 0.0006 | <0.0001 |
| Ala | 2.2 (−2.2 to 6.2) | −2.7 (−7.1 to 0.1) | 2.9 (−1.6 to 6.7) | <0.0001 | 0.0204 | 0.0481 |
| Tau | 0.7 (−0.8 to 2.5) | −1.2 (−3.8 to 2.0) | 2.1 (0.03 to 4.7) | <0.0001 | 0.6612 | <0.0001 |
| Thr | 0.1 (−1.2 to 1.4) | 0.3 (−1.0 to 1.7) | 1.5 (−0.6 to 2.7) | 0.3926 | 0.0921 | 0.4038 |
| Pro | 0.3 (−1.4 to 3.1) | −0.6 (−2.6 to 1.5) | −0.01 (−1.9 to 2.0) | 0.0777 | 0.2217 | 0.6023 |
| Val | 0.8 (−1.9 to 3.1) | −0.7 (−2.9 to 1.4) | 1.0 (−0.2 to 2.9) | <0.0001 | 0.0007 | 0.0185 |
| Met | 0.04 (−0.2 to 0.4) | −0.1 (−0.3 to 0.3) | 0.2 (−0.2 to 0.4) | 0.4806 | 0.0003 | 0.0040 |
| Ile | 0.4 (−0.6 to 1.1) | −0.1 (−0.9 to 0.6) | 0.3 (−0.1 to 1.0) | <0.0001 | 0.1139 | 0.0006 |
| Tyr | 0.4 (−0.4 to 1.3) | −0.4 (−1.3 to 0.4) | 0.7 (−0.2 to 1.1) | <0.0001 | 0.0002 | 0.0090 |
| Leu | 0.7 (−0.6 to 2.2) | −0.2 (−1.6 to 0.9) | 1.3 (0.4 to 2.3) | <0.0001 | 0.8250 | <0.0001 |
| Phe | 0.7 (−0.1 to 1.5) | −0.8 (−1.5 to 0.1) | 1.3 (0.7 to 2.0) | <0.0001 | 0.0994 | <0.0001 |
| Trp | −0.5 (−2.2 to 1.2) | −1.0 (−2.1 to 0.4) | −0.2 (−2.0 to 1.3) | 0.0003 | 0.0062 | <0.0001 |

$p$ < 0.008 (0.05/6 comparisons) was considered statistically significant.

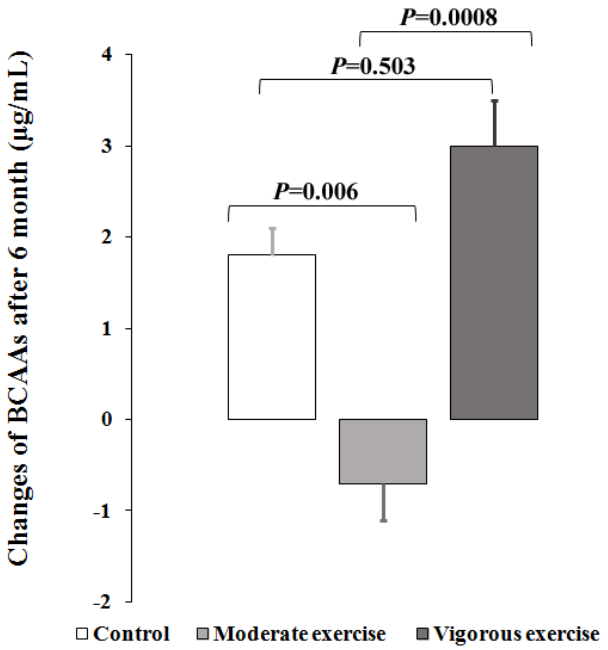


**Figure 3.** The sum of BCAAs concentrations changes after 6 months of moderate and vigorous exercise training.

## 4. Discussions

A simple UHPLC-MS/MS method with a small volume plasma sample for the determination of eighteen AAs in human plasma was developed and validated in our study, and then we analyzed the association between AAs concentrations and clinical characteristics, and assessed AAs changes after long-term exercise training in NAFLD subjects. To our best knowledge, this was the first study that reported the relationship between plasma AAs concentrations and clinical characteristics in NAFLD patients, and compared the effect on AAs alteration following different long-term exercise intensities.

Our study observed that AAs concentrations were closely associated with clinical characteristics in NAFLD, Gly and Ser were inversely correlated with IHTG content, Ala and BCAAs (Val, Ile, Leu) were positively correlated with IHTG content, respectively. Thus, these AAs might be another potential indicator of NAFLD, since IHTC was a diagnostic criterion for NAFLD in the clinic (IHTG content > 5% was considered as NAFLD). Prior studies have shown a close association between BCAAs and HOMA-IR, liver fat 9. Our work indicated a statistically significant association between BCAAs and IHTG content ($r = 0.18$, $p = 0.007$), which verified the vitally important role of BCAAs in NAFLD progression.

In the metabolism of branched-chain amino acids (BCAAs), branched-chain ketoacid dehydrogenase (BCKDH) acts as the rate-limiting enzyme, and its phosphorylation inhibits its activity. Mitochondrial pyruvate carrier (MPC) inhibitors can dephosphorylate BCKDH by activating PPM1K, and also activate the AMP-activated protein kinase (AMPK) and mechanistic target of rapamycin complex 2 (mTORC2) signaling pathways to promote BCAA catabolism and reduce plasma BCAA concentrations. In non-alcoholic steatohepatitis (NASH), the reduction in BCAA concentrations improves insulin resistance. Treatment with MPC inhibitors in NASH patients can reduce plasma BCAAs and improve related metabolic parameters, thereby ameliorating the disease [27]. Ni Y et al. [28] study shows that gut microbiota such as Bacteroides stercoris can synthesize branched-chain amino acids (BCAAs), and their levels are positively correlated with intrahepatic fat and liver enzymes. BCAAs can promote lipid synthesis in hepatocytes, inhibit fatty acid oxidation, and may synergize with lipopolysaccharides (LPS) to exacerbate inflammation. Entering the bloodstream, BCAAs act on the liver, promoting lipid accumulation and triggering inflammation, thereby influencing non-alcoholic fatty liver disease (NAFLD). Resistant starch (RS) can reduce serum BCAAs levels by inhibiting BCAAs-producing microbiota such as Bacteroides stercoris and their synthetic functions, thus improving NAFLD.

Branched-chain amino acids (BCAAs) regulate exercise metabolism and fatigue through multiple pathways. During moderate-intensity exercise, they promote fat oxidation to reduce glycogen consumption, while during high-intensity exhaustion exercise, they enhance carbohydrate oxidation and improve cycling efficiency. By competing with tryptophan, they inhibit serotonin synthesis and reduce blood ammonia accumulation to alleviate central fatigue. Additionally, BCAAs lower post-exercise insulin levels and promote ammonia metabolism to balance energy and amino acid decomposition. Supplementation with BCAAs during exercise can optimize substrate utilization (prioritizing fat followed by carbohydrates), enhance exercise efficiency, and reduce post-exercise fatigue by regulating neurotransmitters and metabolites, thus positively impacting endurance performance [29]. Additionally, studies have shown that upon oral ingestion of branched-chain amino acids (BCAAs), their leucine can be oxidized to generate $CO_2$ or retained for protein synthesis, leading to a leucine retention rate of approximately 60% to promote whole-body protein synthesis [30]. Supplementation with BCAAs after exercise increases leucine retention and enhances protein synthesis, helping to counteract exercise-induced protein breakdown and maintain muscle protein metabolic balance.

After 6 months of moderate exercise training, BCAAs concentrations were decreased; the underlying mechanisms remain uncertain. NAFLD was associated with downregulation of BCAA catabolic enzymes, which contribute to the accumulation of BCAA in plasma [28], might be partially reversed in response to exercise, leading to a decrease of BCAAs in blood. In the hand, exercise training improved the oxidative potential of muscle mitochondria [31], where BCAAs catabolism occurred [32], thus improvement of mitochondrial oxidative capacity would likely impact the processes for consuming excess plasma BCAAs.

Our study found that plasma AAs concentrations changed differently following different exercise intensities. Compared to control, moderate exercise and vigorous exercise showed inversely impact on His, Ser, Gln, Val, Tyr and Trp concentrations changes, which might because muscle metabolic variables were different during various exercise condition: a clinical trial found that in overweight human, some AAs levels were did not change on average with month training program [20,21], in healthy adult males, plasma BCAAs concentrations were decreased by an acute exercise bout [22] or increased after a ten-week standardized exercise program [23]. We hypothesized at first that high-intensity exercise could largely change AAs concentrations, since a recent reported monitored that the strongest response of serum metabolic fingerprint was seen after resistance exercise, followed by high-intensity

interval exercise, while the effect of continuous moderate-intensity exercise was weak in men with metabolic syndrome [33]. However, the results in our work indicated that 6 months of moderate exercise seemed more efficient on AAs metabolism improvement in inconsistent with prior studies, especially on BCAAs decreasing. For now, the inner mechanism of amino acids changes following various intensity exercises were limited, we considered that exercise intensity was an important modulator of plasma AAs profile, and moderate exercise benefits AAs metabolism better than vigorous exercise due to the better impact on the enzymatic and molecular processes regulating AAs delivery and oxidation in liver and/or skeletal muscle, according to the finding that low-intensity exercise favors a fat oxidation rate than in the high intensity exercise group with a greater decrease in body mass and fat mass [34], which needed much more further investigation to confirm these hypothesis.

The major strength of our study was a moderately large number of participants treated with long-term exercise training in different exercise intensities, and a combination with a novel targeted metabolomics study for AAs profile analysis. Nonetheless, we acknowledge several limitations of our study. Firstly, since our study primarily aimed to investigate the benefits of exercise for NAFLD patients, we focused exclusively on this population and did not include healthy controls. No healthy people participated in the clinic trial, thus we did not know whether AAs concentrations in NAFLD subjects after long-term exercise training remained greater or returned to normal levels. In future studies, we may consider including healthy controls to distinguish between exercise-induced AAs changes specific to NAFLD and general physiological responses. Secondly, moderate exercise was more efficient on AAs changes than vigorous exercise, with a lack of inner mechanism, and impact on BCAAs concentrations after exercise in tissues where BCAAs are metabolized (muscle, adipose, and liver) were unknown; thus, a corresponding animal model was necessary to verify the clinical finding.

## 5. Conclusions

Several plasma AAs were closely associated with the main clinical characteristics in NAFLD patients, and altered after 6 months of exercise training. These results reveal differential effects of exercise intensity on AAs metabolism in NAFLD, particularly BCAA reduction with moderate exercise. While this suggests a potential metabolic benefit, whether these changes directly drive NAFLD improvement requires further investigation.

**Supplementary Materials:** The following supporting information can be downloaded at: https://media.sciltp.com/articles/others/2508121548536552/HM-815-Supplementary-Materials.pdf, Figure S1: Chromatogram of eighteen AAs in human plasma on positive ion mode; Table S1: MS/MS conditions and retention time of IS and AAs in positive ion mode; Table S2: Linearity range, LLOD and LLOQ of AAs in human plasma; Table S3: Accuracy, intra-day/inter-day precision, recovery and matrix effect of AAs in human plasma (data were represented as mean ± standard deviation); Table S4: Baseline characteristics of study participants with NAFLD (n=220); Table S5: Clinical characteristics changes after 6 month exercise training on NAFLD participants.

**Author Contributions:** J.L., X.L., X.S. and Z.C. (Zhong Chen) contributed the study conception and design, J.L. and W.S. wrote the manuscript, X.S. was responsible for data analysis, Y.Z., Z.C. (Zheng Chen) ang S.W. were participated in clinical trial study, data analysis and blood sample collection. C.H. and X.L. provided the funding. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the First Affiliated Hospital of Xiamen University (XMYY-2022KY068).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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**Conflicts of Interest:** The authors declare there are no competing interests.

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