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Received: 28 July 2022 | Revised: 5 December 2022 | Accepted: 18 December 2022

DOI: 10.1002/oby.23711

ORIGINAL ARTICLE

Obesity Biology and Integrated Physiology

Obesity A Research Journal | THE OBESITY SOCIETY | WILEY

# Hepatic and serum branched-chain fatty acid profile in patients with nonalcoholic fatty liver disease: A case–control study

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**Funding information**
Institute of Health "Carlos III" (ISCIII), Grant/Award Numbers: CP19/00098, FI21/00003, INT21/00078, PI20/00505

## Abstract

**Objective:** Alterations in the hepatic lipidome are a crucial factor involved in the pathophysiology of nonalcoholic fatty liver disease (NAFLD). The aim of this study was to evaluate the serum and hepatic profile of branched-chain fatty acids (BCFAs) in patients with different stages of NAFLD.

**Methods:** This was a case–control study performed in 27 patients without NAFLD, 49 patients with nonalcoholic fatty liver, and 17 patients with nonalcoholic steatohepatitis, defined by liver biopsies. Serum and hepatic levels of BCFAs were analyzed by gas chromatography–mass spectrometry. The hepatic expression of genes involved in the endogenous synthesis of BCFAs was analyzed by real-time quantitative polymerase chain reaction (RT-qPCR).

**Results:** A significant increase in hepatic BCFAs was found in subjects with NAFLD compared with those without NAFLD; no differences were observed in serum BCFAs between study groups. Trimethyl BCFAs, iso-BCFAs, and anteiso-BCFAs were increased in subjects with NAFLD (either nonalcoholic fatty liver or nonalcoholic

José Ignacio Martínez-Montoro and María Ángeles Núñez-Sánchez contributed equally to this work and share first authorship.

steatohepatitis) compared with those without NAFLD. Correlation analysis showed a relationship between hepatic BCFAs and the histopathological diagnosis of NAFLD, as well as other histological and biochemical parameters related to this disease. Gene expression analysis in liver showed that the mRNA levels of *BCAT1*, *BCAT2*, and *BCKDHA* were upregulated in patients with NAFLD.

**Conclusions:** These results suggest that the increased production of liver BCFAs might be related to NAFLD development and progression.

## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has become the most prevalent cause of chronic liver disease worldwide, leading to major health and socioeconomic consequences [1]. The spectrum of NAFLD ranges from simple steatosis or nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH), which may evolve to fibrosis and cirrhosis in a significant number of patients and ultimately to hepatocellular carcinoma and liver failure [2, 3]. Importantly, the pathogenesis of NAFLD is closely associated with metabolic dysfunction; indeed, this entity usually coexists with other metabolic comorbidities, such as obesity, type 2 diabetes mellitus, insulin resistance, dyslipidemia, or hypertension [4]. Although the mechanisms involved in the development and progression of NAFLD are not fully understood, sedentary lifestyle and excessive caloric intake, obesity, insulin resistance, genetic modifiers, and age have been postulated to play an important role [5]. In addition, emerging factors, such as the gut microbiome and related metabolites, have been reported to critically contribute to the pathogenesis of NAFLD [6].

Recent data suggest that alterations in the hepatic lipidome constitute another crucial factor involved in the pathophysiology of NAFLD [7, 8]. Thus, human liver lipidomics studies have revealed substantial differences in the content of fatty acids (FAs) between healthy controls and patients with NAFLD, including changes in saturated FAs (SFAs), monounsaturated FAs, and polyunsaturated FAs [9–14]. Specifically, NAFLD is characterized by an accumulation of SFAs, which could induce lipotoxicity and insulin resistance in hepatocytes, a fact that may have a major influence on the development and progression of the disease [15, 16]. However, less attention has been paid to specific SFA species, such as branched-chain FAs (BCFAs).

BCFAs are SFAs with one or more methyl branches on the carbon chain (therefore, categorized as mono-, di-, or multimethyl BCFAs), and they are named as iso- or anteiso- depending on their termination (isopropyl and isobutyl, respectively) [17]. As they are primarily found in full-fat dairy and beef products, the dietary intake of BCFAs is the main source of these FAs. However, the endogenous synthesis of BCFAs from their branched-chain amino acid (BCAA) precursors (i.e., valine, leucine, and isoleucine) has also been described [17–19].

Interestingly, BCFAs have been suggested to be associated with a myriad of health benefits including anticarcinogenic effects, obesity prevention, or anti-inflammatory properties [20–26]. For instance, serum levels of BCFAs were reported to be inversely correlated with inflammation, insulin resistance, hypertriglyceridemia, and other characteristics of the metabolic syndrome in subjects with obesity [27].

In mammals, BCFAs have been detected primarily in mammalian meibomian glands [28], but they have also been detected in other tissues including the liver, albeit at much lower levels [19, 29]. In line with this, although some attempts have been made to explore the role that hepatic BCFAs may have in liver health, their relationship with human chronic liver conditions such as NAFLD remains poorly understood [25].

Thus, in the present study, we aimed to evaluate the serum and hepatic BCFA profile in patients with biopsy-proven NAFLD. Furthermore, we investigated whether the serum and hepatic BCFA profile is associated with NAFLD-related risk factors as well as with expression levels of enzymes potentially involved in the endogenous synthesis of BCFAs.

### Study Importance

#### What is already known?

- Nonalcoholic fatty liver disease (NAFLD) has become the most prevalent cause of chronic liver disease worldwide and is associated with metabolic dysfunction.
- Alterations in the hepatic lipidome might play a role in the pathophysiology of NAFLD.

#### What does this study add?

- Circulating branched-chain fatty acids (BCFAs) do not correlate with NAFLD progression.
- Hepatic BCFAs are elevated in patients with simple steatosis and nonalcoholic steatohepatitis compared with subjects without NAFLD.
- Hepatic BCFAs are related to the histopathological diagnosis of NAFLD.

#### How might these results change the direction of research or the focus of clinical practice?

- Given these results, the role of increased BCFAs in liver and their role in NAFLD development and progression should be further investigated.

# METHODS

## Study design and participants

In this study, we included 93 consecutive patients with severe obesity who underwent bariatric surgery at the Virgen de la Arrixaca University Hospital (Murcia, Spain) in 2020 and 2021. Inclusion criteria included a signed informed consent, age 18 to 65 years, body mass index (BMI) ≥40 kg/m$^2$ or ≥35 kg/m$^2$ with significant obesity-related comorbidities, and duration of obesity ≥5 years. Exclusion criteria were evidence of liver disease other than NAFLD (including viral hepatitis, medication-related disorders, autoimmune disease, hepatocellular carcinoma, hemochromatosis, Wilson's disease, familial/genetic causes), a previous history of excessive alcohol use (>30 g daily for men and >20 g daily for women), treatment with any drugs potentially causing steatosis, such as tamoxifen, amiodarone, and valproic acid, or subjects who declined to participate.

Study groups were defined based on the histological evaluation of liver biopsies according to the SAF (Steatosis, Activity, Fibrosis) classification system as (1) patients without NAFLD (liver without any histological alteration); (2) patients with NAFL, defined by the presence of at least grade 1 (5%) liver steatosis, with or without ballooning or lobular inflammation but not both at the same time; and (3) patients with NASH, defined by the presence of at least grade 1 steatosis, ballooning, and lobular inflammation, with or without fibrosis [30].

The study was performed in agreement with the Declaration of Helsinki according to local and national laws and was approved by the Ethics and Clinical Research Committees of the Virgen de la Arrixaca University Hospital (ref. number 2020-2-4-HCUVA).

## Biochemical evaluation

Blood samples were collected from study patients after an overnight fast of at least 12 hours, and serum was separated by centrifugation. The determination of glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and albumin levels was carried out using the Cobas Analyzer c702 (Roche). The levels of glycated hemoglobin (HbA1c) were measured with the glycohemoglobin analyzer HLC-723G8 (Tosoh Bioscience). Insulin levels were measured using the Cobas Analyzer e801 (Roche). Insulin resistance was determined by the homeostasis model assessment of insulin resistance index and was calculated as insulin (μU/mL) × glucose (mmol/L)/22.5 [31].

## Liver biopsies collection and sample processing

Intraoperative wedge liver biopsies of at least 1 cm in depth were obtained from patients who underwent bariatric surgery. One section of the liver biopsy was snap-frozen and stored at −80 °C, and the other was formalin-fixed and paraffin-embedded for histological assessment. Paraffin-embedded 5-μm sections of human liver biopsies were stained for hematoxylin and eosin (H&E), Masson trichrome, Periodic acid–Schiff (PAS), Perls, and reticulin staining. All biopsies were reviewed and scored by trained liver pathologists from the Virgen de la Arrixaca University Hospital to determine the SAF score [30].

## Gas chromatography–mass spectrometry analysis of BCFAs

Total lipids were extracted from whole serum and tissue samples from liver biopsies with a chloroform-methanol mixture (2:1, volume per volume), as described previously [32]. The lipid extracts were dried by evaporation under a stream of nitrogen. FAs are hydrolyzed from total esterified lipids, but the samples also contained FAs that were present in samples in a free form. Each sample was hydrolyzed with 1 mL of 0.5M KOH in methanol at 90 °C for 3 hours. The mixture was acidified with 0.2 mL of 6M HCl and then 1 mL of water was added. Nonesterified FAs were extracted three times with 1 mL of n-hexane and evaporated to dryness under a stream of nitrogen. FA methyl esters (FAMEs) were prepared using 1 mL of 10% boron trifluoride-methanol solution (90 minutes at 558°C); 1 mL of water was added to the reaction mixture, FAMEs were extracted three times with 1 mL of n-hexane, and the solvent was evaporated.

FAMEs were analyzed with gas chromatography–electron ionization mass spectrometry QP-2010 SE (Shimadzu) and separated on a 30-m 0.25-mm i.d. Rtx-5MS (Restek, cat. No. 10223) capillary column (film thickness 0.25 mm). One microliter of sample was injected at a split mode (ratio 20:0). The temperature of injection, ion source, and transfer line were 300 °C, 200 °C, and 300 °C, respectively. Column temperature was set in a range of 60 °C to 300 °C (48°C/min), with helium as the carrier gas at the column head pressure of 60 kPa. Electron energy used for FAME ionization was 70 eV; 19-methylarachidic acid was used as an internal standard. Full scan mode was used with mass scan range m/z 45 to 700. Accurate identification of the FA profile was possible because of the use of reference standards (BCFA standards, Larodan, and 37 FAME Mix, Sigma-Aldrich, cat. no. CRM47885), reference library NIST 2017 and reference compounds (https://www.lipidmaps.org/resources/lipidweb/index.php?page=ms/methylesters.htm) [33]. BCFA levels were calculated based on internal standard signal and expressed as relative abundance. The m/z of molecular ion and fragment ions as well as the retention time for each BCFA is presented in Supporting Information Table S1. In addition, Supporting Information Figures S1 and S2 present mass spectra and chromatograms of isomers of BCFAs.

## Real-time quantitative polymerase chain reaction analysis

Liver biopsies were collected in 1.5-mL nuclease-free tubes containing 1 mL of RNAlater (Sigma), maintained at 4 °C overnight, and then kept at −80 °C until use. Total RNA from human liver biopsies (10–20 mg)

was extracted using Trizol extraction (Life Technologies) followed by a purification step using the GeneJET RNA Purification kit (Thermo Scientific). RNA quantity and purity were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Only samples with absorbance 260/230 and 260/280 ratios between 1.8 and 2.2 were used for gene expression analysis. A total of 1 μg of RNA was reverse transcribed using the High-Capacity RNA-to-cDNA kit (Applied Biosystems) following the manufacturer's instructions. One negative control for RNA and other for reverse transcriptase were included for each transcription reaction. Real-time quantitative polymerase chain reaction (RT-qPCR) amplification was carried out with the Power SYBR Green Master mix (Applied Biosystems) using a Fast 7500 Real-Time instrument (Applied Biosystems). Relative expression levels were analyzed using the $2^{-\Delta CT}$ method, and 18S rRNA was used as housekeeping gene. The amplification setup used was 40 runs in 96-well plates with SYBR green detection format. Product melting curve measurement was performed in continuous acquisition with 1 °C every 30 seconds. The sequences of the primers used in this study were those previously reported and validated by us [27] and can be found in Supporting Information Table S2.

### Statistical analysis

Data were analyzed using the SPSS Statistics 28.0.1.1 software (IBM Corp.). Normal distribution of the study variables was assessed by the Kolmogorov–Smirnov test. The analysis of categorical data was performed by the χ2 test for intragroup differences and the Pearson χ2 test for the intergroup differences. For quantitative data, Kruskal–Wallis one-way ANOVA on ranks was performed to evaluate group differences in those variables without a normal distribution, whereas ANOVA was used in those parameters following a normal distribution, followed by Bonferroni post hoc analysis for the intergroup differences. Relationships between pairs of continuous variables were determined on the basis of the Spearman correlation test. All values are given as mean ± standard deviation (SD). A level of $p < 0.05$ was considered statistically significant. Figures were represented using the Prism 9.0 program (GraphPad Software Inc.).

## RESULTS

### Characteristics of the study population

Data from 93 patients (27 without NAFLD, 49 with NAFL, and 17 with NASH) were analyzed. Clinical, anthropometric, and biochemical characteristics of the participants included in this study are shown in Table 1. The distribution of the recorded histopathological scores, that is, SAF score, steatosis, lobular inflammation, and hepatocellular ballooning, are shown in Table 2. Fibrosis was not detected in any of the analyzed samples.

Participants with NAFL and NASH had significantly higher glucose, HbA1c, and insulin resistance compared with patients without NAFLD. Patients with NAFL presented significantly lower HDL-c and significantly higher systolic blood pressure, insulin, and triglycerides compared with patients without NAFLD. Finally, serum levels of AST and ALT were higher in subjects with NASH compared with participants with NAFL or without NAFLD.

### Serum and hepatic BCFA composition according to histopathological diagnosis

Serum and hepatic concentrations of the different BCFAs according to histopathological diagnosis (no NAFLD, NAFL, and NASH) are shown in Table 3, Figure 1, and Supporting Information Table S3. No differences among groups were observed regarding total serum concentrations of BCFAs, serum trimethyl BCFAs, serum iso-BCFAs, or serum anteiso-BCFAs (Supporting Information Table S3). However, different concentrations of both total and distinct species of hepatic BCFAs, that is, trimethyl, iso-, and anteiso-BCFAs, were observed between subjects without NAFLD and subjects with NAFL/NASH (Table 3 and Figure 1). Moreover, hepatic concentrations of 2,6,10-trimethyl-12:0 and anteiso-12-M-14:0 BCFAs significantly increased from subjects with NAFL to NASH.

### Correlations between hepatic and serum BCFAs and histopathological/biochemical parameters

Correlations between hepatic and serum BCFAs and different histopathological and biochemical parameters are shown in Table 4 and Supporting Information Tables S4 and S5. A positive correlation was observed between hepatic concentrations of total BCFAs and different histopathological and biochemical parameters related to NAFLD pathogenesis, including histopathological diagnosis, SAF score, steatosis, hepatocyte ballooning, lobular inflammation, HbA1c, insulin, homeostasis model assessment of insulin resistance, serum triglycerides, serum levels of AST, and serum levels of ALT. Similarly, positive correlations between hepatic trimethyl BCFAs/iso-BCFAs/anteiso-BCFAs and these histopathological and biochemical variables were observed (Table 4). Associations between histopathological parameters and specific subtypes of hepatic trimethyl BCFAs/iso-BCFAs/anteiso-BCFAs are shown in Supporting Information Table S4. No associations were found between serum BCFA levels and the NAFLD-related histopathological and biochemical parameters (Supporting Information Table S5).

### Gene expression of enzymes involved in BCFA metabolism

The expression of genes described to be involved in BCFA synthesis was analyzed in liver tissue samples (Figure 2). The levels of BCAA transaminases 1 and 2 (*BCAT1* and *BCAT2*) were significantly

**TABLE 1** Characteristics of the study population

| | No NAFLD ($n = 27$) | NAFL ($n = 49$) | NASH ($n = 17$) | $p$ value |
|---|---|---|---|---|
| Gender (F/M) | 26/1 | 32/17 | 14/3 | 0.007 |
| Age (y) | 44.7 ± 11.7 | 47.8 ± 11.2 | 48.4 ± 9.9 | 0.444 |
| Weight (kg) | 113.9 ± 11.5 | 115.8 ± 20.3 | 118.4 ± 23.2 | 0.884 |
| BMI (kg/m$^2$) | 42.0 ± 4.6 | 43.0 ± 6.0 | 44.2 ± 6.0 | 0.437 |
| WC (cm) | 119.5 ± 8.6 | 126.4 ± 12.4 | 127.1 ± 16.6 | 0.052 |
| SBP (mmHg) | 130.9 ± 17.0$^a$ | 143.5 ± 18.1$^b$ | 141.4 ± 22.3$^{ab}$ | 0.024 |
| DBP (mmHg) | 81.5 ± 11.2 | 86.4 ± 9.7 | 81.5 ± 10.5 | 0.131 |
| Glucose (mg/dL) | 90.6 ± 9.7$^a$ | 109.1 ± 35.9$^b$ | 123.8 ± 63.6$^b$ | 0.022 |
| HbA1c (%) | 5.6 ± 0.7$^a$ | 6.2 ± 1.2$^b$ | 6.5 ± 2.0$^b$ | 0.018 |
| Insulin (μIU/mL) | 13.3 ± 12.2$^a$ | 18.0 ± 18.1$^b$ | 19.0 ± 14.1$^{a,b}$ | 0.029 |
| HOMA-IR | 3.0 ± 2.7$^a$ | 5.0 ± 6.3$^b$ | 5.6 ± 5.1$^b$ | 0.003 |
| Cholesterol (mg/dL) | 158.3 ± 27.4 | 169.2 ± 34.0 | 152.5 ± 24.1 | 0.113 |
| HDL-c (mg/dL) | 48.7 ± 11.4$^a$ | 40.7 ± 10.7$^b$ | 41.6 ± 11.5$^{a,b}$ | 0.003 |
| LDL-c (mg/dL) | 84.0 ± 23.2 | 89.8 ± 33.0 | 77.6 ± 23.1 | 0.341 |
| Triglycerides (mg/dL) | 135.7 ± 40.5$^a$ | 229.9 ± 183.7$^b$ | 174.9 ± 86.4$^{ab}$ | 0.023 |
| AST (U/L) | 16.9 ± 4.8$^a$ | 20.5 ± 7.6$^a$ | 28.9 ± 12.5$^b$ | 0.001 |
| ALT (U/L) | 15.6 ± 6.1$^a$ | 23.4 ± 11.1$^a$ | 37.0 ± 18.5$^b$ | 0.001 |
| Albumin (g/dL) | 4.0 ± 0.4 | 4.2 ± 0.4 | 4.2 ± 0.4 | 0.497 |
| Platelets (10$^3$/μL) | 243.0 ± 61.6 | 257.6 ± 60.8 | 274.8 ± 89.0 | 0.451 |

*Note*: Values are presented as mean ± SD. *P* values were calculated using ANOVA in those parameters with a normal distribution (i.e., age, BMI, WC, SBP, cholesterol, LDL-c, and platelets), considering $p < 0.05$ significant; whereas $p$ values for parameters without a normal distribution were calculated using a Kruskal–Wallis test (considering $p < 0.05$ significant), followed by a Bonferroni post hoc analysis for the intergroup differences test in those parameters with a significant $p$ value. Different superscript letters indicate statistically significant difference at $p < 0.05$ within each row between the groups.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBP, diastolic blood pressure; F, female; HbA1c, glycated hemoglobin; HDL-c, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-c, low-density lipoprotein cholesterol; M, male; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; SBP, systolic blood pressure; WC, waist circumference.

**TABLE 2** Liver histopathological findings in the study population

| | NAFL ($n = 49$) | NASH ($n = 17$) | $p$ value |
|---|---|---|---|
| SAF score (0/1/2/3/4/5/6/7) | (0/15/19/11/4/0/0/0) | (0/0/0/4/9/2/1/1) | <0.001 |
| Steatosis (0/1/2/3) | (0/31/15/3) | (0/7/7/3) | 0.189 |
| Hepatocellular ballooning (0/1/2) | (18/30/1) | (1/11/5) | <0.001 |
| Lobular inflammation (0/1/2/3) | (49/0/0/0) | (0/14/3/0) | <0.001 |

*Note*: Values are presented as frequencies. *P* values were calculated using Pearson χ2 test considering $p < 0.05$ as significant.
Abbreviations: NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; SAF, Steatosis, Activity and Fibrosis.

**TABLE 3** BCFAs quantified in liver samples

| | No NAFLD ($n = 27$) | NAFL ($n = 49$) | NASH ($n = 17$) |
|---|---|---|---|
| Total hBCFAs | 0.351 ± 0.164$^a$ | 0.780 ± 0.654$^b$ | 0.992 ± 0.813$^b$ |
| hTriM-BCFAs | 0.028 ± 0.019$^a$ | 0.051 ± 0.046$^b$ | 0.075 ± 0.049$^b$ |
| hIso-BCFAs | 0.143 ± 0.047$^a$ | 0.291 ± 0.235$^b$ | 0.356 ± 0.283$^b$ |
| hAnteiso-BCFAs | 0.178 ± 0.110$^a$ | 0.437 ± 0.393$^b$ | 0.560 ± 0.497$^b$ |

*Note*: Values are expressed as relative abundance (mean ± SD). Statistical differences were calculated using the Kruskal–Wallis test (considering $p < 0.05$ significant), followed by a Bonferroni post hoc analysis for the intergroup differences test in those parameters with a significant $p$ value. Different superscript letters indicate statistically significant difference at $p < 0.05$ within each row between the groups.
Abbreviations: BCFAs, branched-chain fatty acids; hAnteiso-BCFAs, hepatic anteiso-BCFA representing the sum of all hAnteiso- species determined; hBCFAs: hepatic BCFAs; hIso-BCFA, hepatic iso-BCFAs representing the sum of all hIso- species determined; hTriM-BCFAs, hepatic trimethyl BCFAs representing the sum of all hTriM- species determined; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

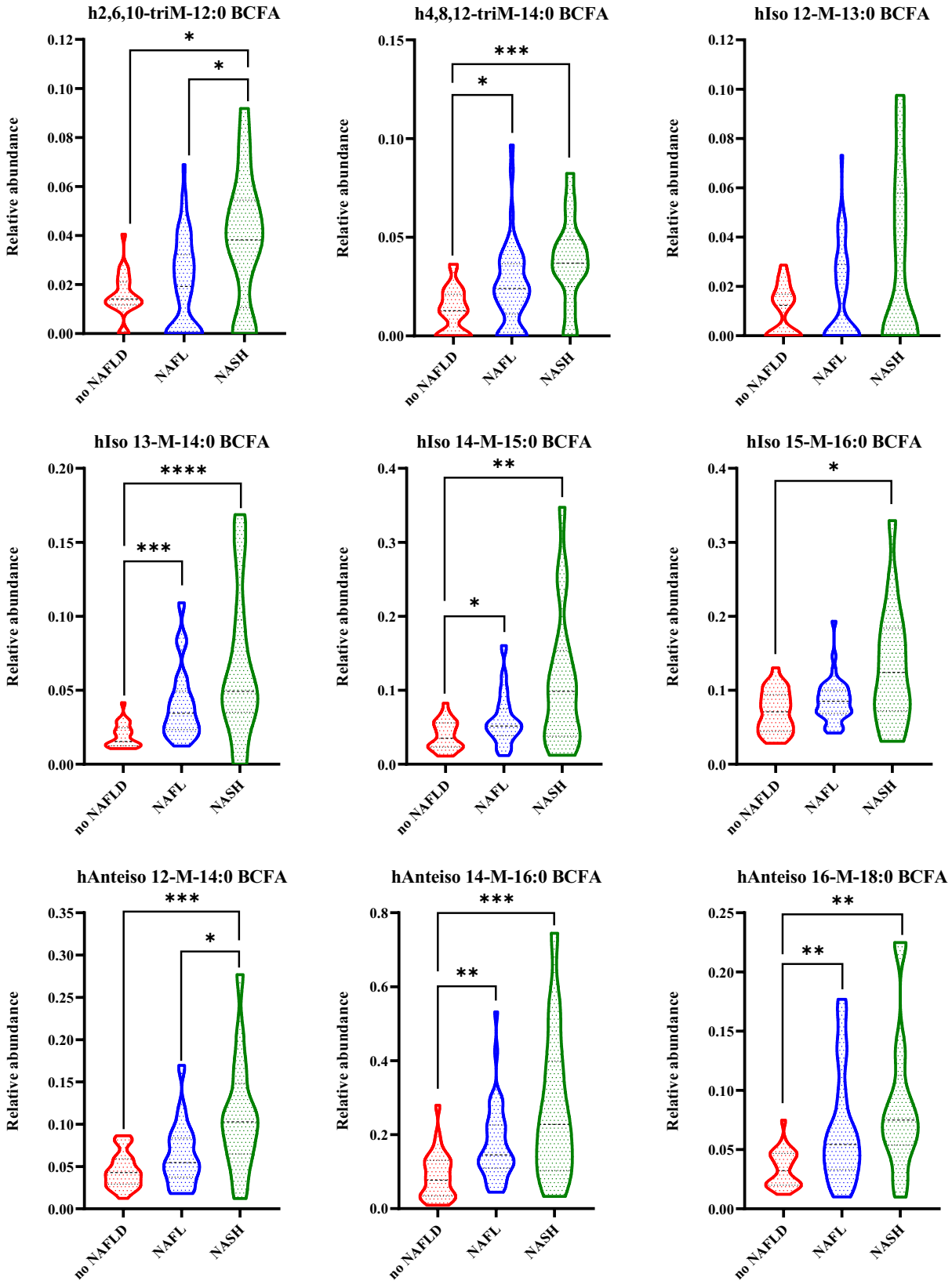


**FIGURE 1** Hepatic levels of individual BCFAs quantified by gas chromatography-mass spectometry. Represented as relative abundance. Statistical differences were calculated using the Kruskal–Wallis test (considering $p < 0.05$ significant), followed by a Bonferroni post hoc analysis for the intergroup differences test in those parameters with a significant $p$ value. BCFAs, branched-chain fatty acids; h, hepatic; NAFLD, nonalcoholic fatty liver disease; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; triM, trimethyl. Medians and quartiles are represented by black and colored lines, respectively, in violin plots. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

**TABLE 4** Correlations between hepatic BCFAs and histopathological/biochemical parameters

| | hBCFAs | hTriM-BCFAs | hIso-BCFAs | hAnteiso-BCFAs |
|---|---|---|---|---|
| Histopathological diagnosis | 0.447** | 0.396** | 0.372** | 0.462** |
| SAF score | 0.487** | 0.416** | 0.432** | 0.494** |
| Steatosis | 0.551** | 0.456** | 0.494** | 0.561** |
| Hepatocyte ballooning | 0.254* | 0.192 | 0.242* | 0.252* |
| Lobular inflammation | 0.243* | 0.306** | 0.172 | 0.246* |
| HbA1c (%) | 0.242* | 0.192 | 0.218* | 0.226* |
| Insulin (μIU/mL) | 0.253* | 0.206 | 0.202 | 0.263* |
| HOMA-IR | 0.321** | 0.256* | 0.278** | 0.329** |
| AST (U/L) | 0.481** | 0.379** | 0.417** | 0.481** |
| ALT (U/L) | 0.584** | 0.356** | 0.513** | 0.595** |
| Triglycerides (mg/dL) | 0.294** | 0.314** | 0.256* | 0.286* |

*Note*: Values are presented as Spearman correlation coefficient. No correction for multiple comparisons was performed; therefore, an increased risk for type I error may be expected.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; h, hepatic; hAnteiso-BCFAs, hepatic anteiso-BCFA representing the sum of all hAnteiso- species determined; HbA1c, glycated hemoglobin; hBCFAs, hepatic branched-chain fatty acids; hIso-BCFA, hepatic iso-BCFAs representing the sum of all hIso- species determined; HOMA-IR, homeostatic model assessment of insulin resistance; hTriM-BCFAs, hepatic trimethyl BCFAs; SAF, Steatosis, Activity and Fibrosis.

**$p$ <0.01.

*$p$ <0.05.

upregulated in patients with NASH compared with patients with NAFL ($p = 0.024$ and $p = 0.046$, respectively) or without NAFLD ($p = 0.031$ and $p = 0.033$, respectively).

In addition, the expression of the branched chain keto acid dehydrogenase E1 subunit alpha (*BCKDHA*) was significantly increased in the NASH group compared with those patients without NAFLD ($p = 0.031$) but not compared with patients with NAFL. Finally, no differences were observed among the groups in the expression levels of *BCKDHB* (Figure 2).

Interestingly, significant correlations were observed between gene expression of some of the enzymes involved in BCFA metabolism and hepatic/serum BCFAs (Supporting Information Table S6).

## DISCUSSION

In the present study, we evaluated the profile of BCFAs in both serum and liver tissue samples of individuals with severe obesity and with different grades of biopsy-proven NAFLD. Despite the fact that we found no differences in the serum profile of BCFAs between the studied groups, we observed a strong positive correlation between the levels of hepatic BCFAs not only with the histopathological diagnosis of NAFLD but also with other histopathological and biochemical parameters associated with NAFLD such as SAF score, hepatocyte ballooning, or the levels of AST and ALT. Remarkably, this study is the first one to describe that the levels of hepatic BCFAs are altered in patients with NAFLD (either NAFL or NASH) compared with those without NAFLD.

Our detailed analysis of the specific types of BCFAs evaluated in liver tissue (trimethyl, iso-, and anteiso-BCFAs) also showed that the hepatic levels of 4,8,12-trimethyl-14:0, iso-13-M-14:0, iso-14-M-15:0, anteiso-14-M-16:0, and anteiso-16-M-18:0 BCFAs were significantly elevated in the NAFLD groups (NAFL and NASH) compared with the no NAFLD group. Furthermore, the NASH group also had increased hepatic levels of 2,6,10-trimethyl-12:0, iso-15-M-16:0, and anteiso-12-M-14:0 BCFAs compared with the no NAFLD group. It is noteworthy that the differences observed in the levels of hepatic 2,6,10-trimethyl-12:0 and anteiso-12-M-14:0 BCFAs were not only significant between the no NAFLD and the NASH groups but also between the NAFL and the NASH groups. These results suggest that some BCFAs might be involved in the development and progression of NAFLD, although further investigation is needed.

In our study, we also assessed the associations between the profile of BCFAs in both serum and liver samples, and NAFLD occurrence and severity in patients with severe obesity were also investigated. Although some differences could be expected regarding the circulating levels of BCFAs between subjects with NAFLD and without NAFLD, our results showed that there were no significant differences between them. Recent studies have shown that circulating levels of BCFAs were reduced in individuals with obesity compared with lean subjects [27, 32, 34]. In this study, all the participants were patients with severe obesity, and this could explain the lack of significant differences in serum samples. On the other hand, it is also known that diet is an important source of BCFAs in humans, and potential interindividual differences with regard to dietary intake in our population might also play a role in these findings [17]. Nevertheless, in line with the aforementioned results, it could be suggested that BCFAs behave differently in serum and liver tissues. Thus, circulating BCFAs have been reported to have antiobesogenic and anti-inflammatory effects [23, 32], whereas less evidence is available regarding the role of BCFA

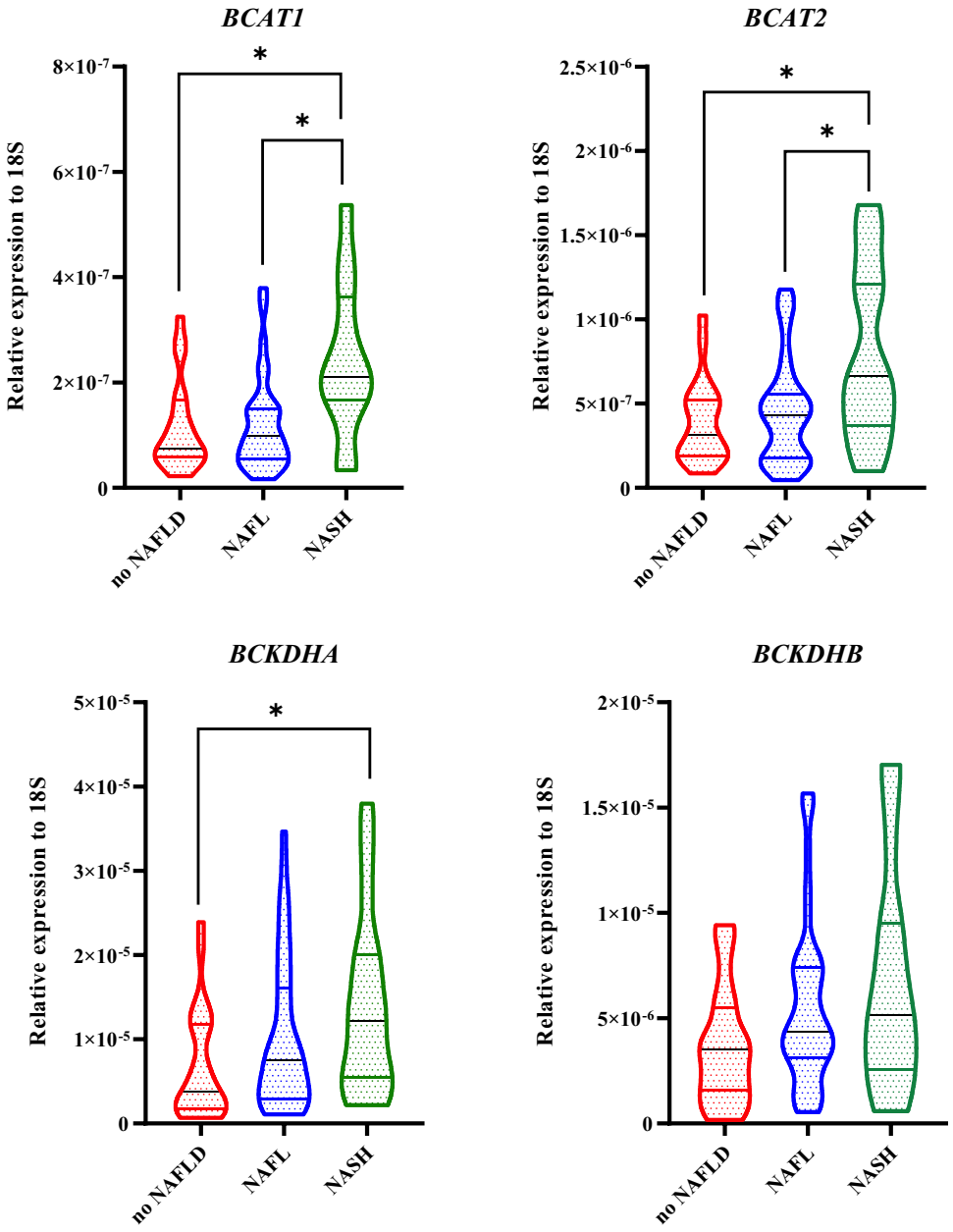


**FIGURE 2** Hepatic expression of genes involved in branched-chain fatty acid synthesis. The results are represented as the fold change of the NAFL and NASH groups, with respect to the no-NALFD group. Statistical analyses were performed using the Kruskal–Wallis test followed by a Bonferroni post hoc analysis for the intergroup differences test in those parameters with a significant *p* value. *BCAT1*, branched-chain amino acid transaminase 1; *BCAT2*, branched-chain amino acid transaminase 2; *BCKDHA*, branched-chain acid dehydrogenase E1 subunit alpha; *BCKDHB*, branched-chain acid dehydrogenase E1 subunit beta; NAFLD, nonalcoholic fatty liver disease; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis. All values are presented as mean ± SD. $^{*}p < 0.05$

concentrations in other tissues, such as the liver. In this respect, in a rodent model, García-Carballo et al. suggested an antisteatotic role of BCAA-derived monomethyl BCFAs in the context of a high-protein diet [25]. On the other side, other works have reported liver-induced damage by some BCFAs [35, 36].

The presence of BCFAs in mammalian tissue is known to be low. It is estimated that they represent about 1% to 2% of the total FA pool [17], and they are primarily derived from microbial metabolism or through dietary dairy products and ruminant meat intake [37]. However, evidence showed that BCFAs may also be synthesized endogenously from their BCAA precursors, that is, valine, leucine, and isoleucine [25]. Furthermore, some studies have described that the decrease in levels of serum BCFAs is inversely associated with serum BCAA levels, suggesting that such alterations are related to disturbances in the BCAA catabolism pathway [38, 39]. Another study, done in patients with severe obesity, demonstrated that the expression of genes encoding BCKDH and BCAT (the enzymes responsible for conversion of BCAAs to BCFAs) was reduced in visceral adipose tissue in these individuals compared with lean patients [27]. However, little is known regarding the expression of these genes in human liver

tissue. *In vitro* and *in vivo* studies using animal models have demonstrated that the expression of these genes is different depending on the type of tissue analyzed, highlighting a potential tissue-specific role of BCAA-catabolizing enzymes [40]. In the case of NAFLD, the expression of *BCAT1* is known to be overexpressed in liver [41, 42], and it was described to be associated with higher NAFLD activity scores, steatosis, lobular inflammation, ballooning, and even adverse outcomes [43]. In this sense, our results showed that expression of not only *BCAT1* but also *BCAT2* was significantly increased in subjects without NAFLD or with NAFL compared with those subjects with NASH. Although the mechanisms by which BCAT activity is associated with NAFLD pathogenesis are not well characterized, BCAA metabolism via these enzymes could lead to an imbalance between glutamate and α-ketoglutarate, leading to the accumulation of glutamate [43], which might be related to NAFLD progression [44]. Interestingly, our results also revealed an increase in the expression levels of *BCKDHA*, which disagrees with previous observations made by others [42]. However, this could be related to the characteristics of our study population, as all the participants in this study were patients with severe obesity, who have been described to overexpress genes involved in BCAA catabolism [45]. Overall, the disturbances in the BCAA catabolic pathway enzymes observed in NAFLD groups might be a response mechanism to hepatic stress to compensate imbalances in the liver metabolic profile [46, 47]. Therefore, it could be speculated that the observed differences in hepatic BCFA concentrations among groups may be driven by several enzymes involved in BCAA metabolism, which might play a role in the pathogenesis of NAFLD. In fact, we found some correlations between these enzymes and hepatic BCFA concentrations. However, because of the cross-sectional nature of this study, it cannot be ruled out that the elevation of different hepatic BCFA concentrations in NAFL/NASH may not be a cause but a result of greater disease severity. In addition, other mechanisms, such as the production of BCFAs by the gut microbiome, need to be evaluated in future studies, given the strong association between the gut microbiome and the pathogenesis of NAFLD [6]. Accordingly, potential differences in the synthesis of BCFAs depending on specific patterns of gut microbiota composition in subjects with/without NAFLD could also explain some of our results. Thus, devoted prospective studies are warranted to further assess these hypotheses.

Our study has certain limitations but also some important strengths. The limitations include the relatively small sample size, which may not be enough for detecting differences between low-concentration BCFAs that may be of relevance or to assess overall differences between controls and patients with NAFLD. Another limitation is the inherent nature of the study, a cross-sectional design, wherein only an association and not a cause can be inferred. In addition, our study has the limitation (as do the vast majority of bariatric surgery studies) of gender unbalance, with a clear predominance of women. Also, the gender distribution across the NAFLD spectrum was not homogeneous, precluding us from evaluating gender-specific associations. On the other hand, the strengths of our study lie in the careful design, the definition of NAFLD by liver biopsies, and the determination of serum and hepatic levels of BCFAs by gas chromatography–mass spectrometry.

In conclusion, in this study, we have shown for the first time that NAFLD is associated with significantly increased levels of hepatic BCFAs in patients with severe obesity. Also, we have described that this increase is not reflected in serum levels of BCFAs, as we did not find differences among study groups. Moreover, our findings suggest that the levels of hepatic BCFAs may be influenced by alterations in the enzymes involved in BCAA catabolism. Therefore, changes in hepatic BCFA levels could be associated with the development, progression, and severity of NAFLD, although the exact mechanisms underlying such a relationship still remain to be elucidated.

## AUTHOR CONTRIBUTIONS

José Ignacio Martínez-Montoro, María Ángeles Núñez-Sánchez, Tomasz Sledzinski, Adriana Mika, and José Carlos Fernández-García: data analysis and manuscript preparation. María Ángeles Núñez-Sánchez and María Antonia Martinez-Sanchez: data and sample collection and gene expression analysis. Andrés Balaguer-Román, María Dolores Frutos, Virginia E. Fernández-Ruiz, and Mercedes Ferrer-Gómez: data and sample collection. Tomasz Sledzinski and Adriana Mika: gas chromatography–mass spectrometry analysis. Bruno Ramos-Molina: study design, data analysis, manuscript preparation, and funding acquisition.

## ACKNOWLEDGMENTS

We are particularly grateful for the generous contribution of the patients and the collaboration of Biobank Network of the Region of Murcia, BIOBANC-MUR, registered on the Registro Nacional de Biobancos with registration number B.0000859. BIOBANC-MUR was supported by the Instituto de Salud Carlos III (proyecto PT20/00109), by Instituto Murciano de Investigación Biosanitaria Virgen de la Arrixaca, Biomedical Research Institute of Murcia (IMIB), and by Consejería de Salud de la Comunidad Autónoma de la Región de Murcia.

## FUNDING INFORMATION

This work was funded by the Institute of Health "Carlos III" (ISCIII) and cofunded by the Fondo Europeo de Desarrollo Regional-FEDER (grant number PI20/00505). J.C.F-G was supported by an intensification research program (INT21/00078, ISCIII, Spain; cofunded by the Fondo Europeo de Desarrollo Regional-FEDER), M.A.M-S was supported by a PFIS predoctoral fellowship from the ISCIII (FI21/00003, ISCIII, Spain; cofunded by the Fondo Europeo de Desarrollo Regional-FEDER), and B.R-M was supported by the "Miguel Servet Type I" program (CP19/00098, ISCIII, Spain; cofunded by the Fondo Europeo de Desarrollo Regional-FEDER). The funding organizations played no role in the design of the study, review and interpretation of the data, or final approval of the manuscript.

## CONFLICT OF INTEREST

The authors declared no conflict of interest.

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**How to cite this article:** Martínez-Montoro JI, Núñez-Sánchez María Ángeles, Martinez-Sanchez MA, et al. Hepatic and serum branched-chain fatty acid profile in patients with nonalcoholic fatty liver disease: A case–control study. *Obesity (Silver Spring)*. 2023;31(4):1064-1074. doi:10.1002/oby.23711