Biomedicine & Pharmacotherapy 135 (2021) 111181

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

Branched-chain amino acids and L-carnitine attenuate lipotoxic hepatocellular damage in rat cirrhotic liver

Yasuyuki Tamai [a], Zhen Chen [b], Yue Wu [b], Jun Okabe [c], Yoshinao Kobayashi [a,d], Hitoshi Chiba [e], Shu-Ping Hui [b], Akiko Eguchi [a,f,*], Motoh Iwasa [a,*], Masaaki Ito [g], Yoshiyuki Takei [a]

[a] Department of Gastroenterology and Hepatology, Graduate School of Medicine, Mie University, Tsu, Japan
[b] Faculty of Health Sciences, Hokkaido University, Sapporo, Japan
[c] Epigenetics in Human Health and Diseases, Department of Diabetes, Central Clinical School, Monash University, Australia
[d] Center for Physical and Mental Health, Graduate School of Medicine, Mie University, Tsu, Japan
[e] Department of Nutrition, Sapporo University of Health Sciences, Sapporo, Japan
[f] PRETO, JST, Saitama, Japan
[g] Department of Cardiology and Nephrology, Graduate School of Medicine, Mie University, Tsu, Japan

ARTICLE INFO

Keywords:
L-carnitine
Branched-chain amino acids
Cirrhosis
Lipid peroxides
Cardiolipins

ABSTRACT

Branched-chain amino acids (BCAA) reverse malnutrition and L-carnitine leads to the reduction of hyperammonemia and muscle cramps in cirrhotic patients. BCAA and L-carnitine are involved in glucose and fatty acid metabolism, however their mechanistic activity in cirrhotic liver is not fully understood. We aim to define the molecular mechanism(s) and combined effects of BCAA and L-carnitine using a cirrhotic rat model. Rats were administered carbon tetrachloride for 10 weeks to induce cirrhosis. During the last 6 weeks of administration, cirrhotic rats received BCAA, L-carnitine or a combination of BCAA and L-carnitine daily via gavage. We found that BCAA and L-carnitine treatments significantly improved hepatocellular function associated with reduced triglyceride level, lipid deposition and adipophilin expression, in cirrhotic liver. Lipidomic analysis revealed dynamic changes in hepatic lipid composition by BCAA and L-carnitine administrations. BCAA and L-carnitine globally increased molecular species of phosphatidylcholine. Liver triacylglycerol and phosphatidylcholine hydroperoxides were significantly decreased by BCAA and L-carnitine. Furthermore, serum and liver ATP levels were significantly increased in all treatments, which were attributed to the elevation of mature cardiolipins and mitochondrial component gene expressions. Finally, BCAA and L-carnitine dramatically reduced hepatocellular death. In conclusion, BCAA and L-carnitine treatments attenuate hepatocellular damage through the reduction of lipid peroxides and the overall maintenance of mitochondrial integrity within the cirrhotic liver. These effectiveness of BCAA and L-carnitine support the therapeutic strategies in human chronic liver diseases.

1. Introduction

Liver cirrhosis, the end stage of chronic liver disease, defines by the presence of regenerating nodules and progresses to a decompensated disease stage associating with ascites and hepatic encephalopathy. Liver cirrhosis carries the risk of progressing to liver failure eventually, which is a required factor to be considered for liver transplantation. Several events including increase of oxidative stress [1] and lipid accumulation [2], as well as decrease of hepatocyte number [3], are involved in the progression of cirrhosis. Changes in hepatic lipid metabolism are great

Abbreviations: BCAA, branched-chain amino acids; 4-HNE, 4-hydroxynonenal; PCOOH, phosphatidylcholine hydroperoxides; TGOOH, triglyceride hydroperoxides; HPLC, high performance liquid chromatography; LC/HR-MS/MS, high performance liquid chromatography combined with high-resolution Orbitrap tandem mass spectrometry; CCl_4, carbon tetrachloride; TG, triglyceride; HOMA-R, homeostasis model assessment insulin resistance; ATP, adenosine 5′-triphosphate; dUTP, deoxyuridine triphosphate; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; Tfam, mitochondrial transcription factor A; CPT, Carnitine palmitoyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; LysoPE, lysophosphatidylethanolamine; CL, cardiolipin; LDL, low density lipoprotein; VLDL, very low density lipoprotein; acyl-CoA, acyl coenzyme A; TCA, tricarboxylic acid; B2m, *beta 2 microglobulin.*

* Corresponding authors at: Department of Gastroenterology and Hepatology, Graduate School of Medicine, Mie University, 2-174 Edobashi, Tsu, Mie, 514-8507, Japan.

E-mail addresses: akieguchi@clin.medic.mie-u.ac.jp (A. Eguchi), motoh@clin.medic.mie-u.ac.jp (M. Iwasa).

https://doi.org/10.1016/j.biopha.2020.111181
Received 8 October 2020; Received in revised form 10 December 2020; Accepted 26 December 2020
Available online 1 January 2021

indicator in the progression of liver diseases. The phosphatidylcholine (PC) / phosphatidylethanolamine (PE) molar ratio is a key determination of liver health in several mouse models and human studies; the hepatic PC/PE ratio is between 1.5 and 2.0 for normal liver, while it is below 1.0 for liver failure [4]. Phospholipids control *de novo* lipogenesis by activation of sterol regulatory element-binding proteins (SREBPs), and *vice versa* [5]. Indeed, reduced PC level increases triglyceride (TG) level in the mice with deficiency of PC synthesis [6]. Lysophosphatidlyehanolamine (LysoPE) in a family of lysophospholipids also plays an important role for hepatocellular protection with anti-apoptotic effect [7,8]. Lipid peroxidation occurs as a result of reactive oxygen species (ROS) produced by fatty acid metabolism within the mitochondria and can initiate liver diseases [9] including cirrhosis [10,11]. Oxidized triglyceride rich-low density lipoprotein (LDL) was observed to be increased in the blood of cirrhotic patients, even though total LDL levels were decreased in cirrhotic patients [12], suggesting that lipid peroxides are involved in the progression of chronic liver diseases. The direct analysis of the original lipid peroxides in biological samples would be superior based on the potential to assess a detailed description of their structural diversity as the disease progresses. Indeed, we were able to use the aforementioned lipid peroxides as biomarkers based upon their degree of oxidation [12,13] by our established method of lipid peroxides using high performance liquid chromatography (HPLC) combined with high-resolution Orbitrap tandem mass spectrometry (LC/HR-MS/MS).

Several approaches including anti-viral treatment for viral hepatitis, abstaining from alcohol for alcoholic liver disease, and/or nutritional therapy is recommended to prevent the progression of liver cirrhosis [14–16]. Malnutrition is an independent predictor of adverse clinical outcomes, including survival, in patients with liver cirrhosis [17] and is closely related to complications of decompensation [14,18]. The administration of sufficient protein-rich nourishment and a late evening snack are standard nutritional therapy [19] and nutritional supplementation, such as branched chain amino acids (BCAA) and L-carnitine, are currently recognized as effective treatments for liver cirrhosis [14, 20]. BCAA comprise three essential amino acids: leucine, isoleucine, and valine. BCAA are catalyzed into acyl-coenzyme A (acyl-CoA) derivatives by the branched chain alpha-keto acid dehydrogenase complex, thus granting access to the tricarboxylic acid (TCA) cycle. BCAA have been used as a supplemental therapy to improve malnutrition in liver cirrhotic patients [21,22] that recommended by the European Association for the Study of the Liver (EASL) [14] and the Japan Society of Hepatology (JSH) [23] as the clinical practice guidelines on nutrition in chronic liver disease. Several clinical studies have demonstrated that long-term oral administration of BCAA reduced complications, improved survival curves and increased the overall quality of life in cirrhotic patients [22,24]. We also previously reported that BCAA administration prolonged survival in cirrhotic rats as a result of a decrease in oxidative stress and iron accumulation within the cirrhotic liver [25]. L-carnitine, a vitamin-like constituent of protein, is involved in the transport of fatty acids into the outer mitochondria using carnitine palmitoyltrasnferase I (CPTI) and in the reconversion of the long-chain acylcarnitines to their respective acyl-CoA derivative by CPTII, which is required for fatty acid β oxidation with functional acyl-CoA [26]. L-carnitine deficiency leads to the impaired catabolism of fatty acids resulting in the manifestation of several symptoms, such as hyperammonemia and hepatic encephalopathy [26]. Endogenous carnitine is mainly synthesized from lysine and methionine in the liver, thus one may become carnitine deficient due to a reduction in liver function as a result of hepatocellular damage from cirrhosis [27]. Recent studies have shown the benefits of L-carnitine supplementation in cirrhotic patients with an improvement of refractory hepatic encephalopathy [28,29]. In recent clinical study for chronic liver diseases, some cirrhotic patients have combined effects of BCAA and L-carnitine for the suppression of low albumin concentration and hyperammonemia [30]. However, detailed molecular mechanism of this combined effect is not understood yet.

We hypothesized that BCAA and L-carnitine could improve liver cirrhosis through the metabolism and peroxidization of lipids. In this study, we investigated the molecular mechanism(s) and combined effects of BCAA and L-carnitine supplementation using a carbon tetrachloride-based liver cirrhosis rat model.

2. Methods

2.1. Animal samples

Our animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Hokudo Co., Ltd (Sapporo, Japan). The rat model of liver cirrhosis has been previously described in detail [25]. Briefly, Wister male rats (SPF, CLEA Japan: Tokyo, Japan) aged 7 weeks were fed solid normal diet, CE-2 (CLEA Japan), under conventional condition and were orally administered carbontetrachloride (CCl_4) at 1.0 mL/kg twice a week for 4 weeks to induce liver cirrhosis, at which point the animals were divided into 4 groups by weight stratified random sampling. The cirrhotic rats then received daily administration of either L-carnitine (0.04 g/kg/day) (n = 10), BCAA (10 g/kg/day) (n = 10), L-carnitine plus BCAA combination (L-carnitine 0.04 g/kg/day, BCAA 10 g/kg/day) (n = 10), or 0.9 % saline solution (control) (n = 10). The rat was maintained individually at a constant temperature (23 ± 3 °C), 50 ± 20 % relative humidity and 12 *h light–dark cycles (lights on at 7* a.m.*)*, and had free access to food and water. The cirrhotic state was maintained with twice weekly administration of CCl_4 at 0.5 mL/kg for 6 weeks (10 weeks total) (Fig. 1A). Analysis of rat number was 9/10 in L-carnitine group, 7/10 in BCAA group, 8/10 in L-carnitine plus BCAA combination, and 8/10 in control group due to death by CCl_4 in the experimental term.

2.2. Liver and blood sample preparation

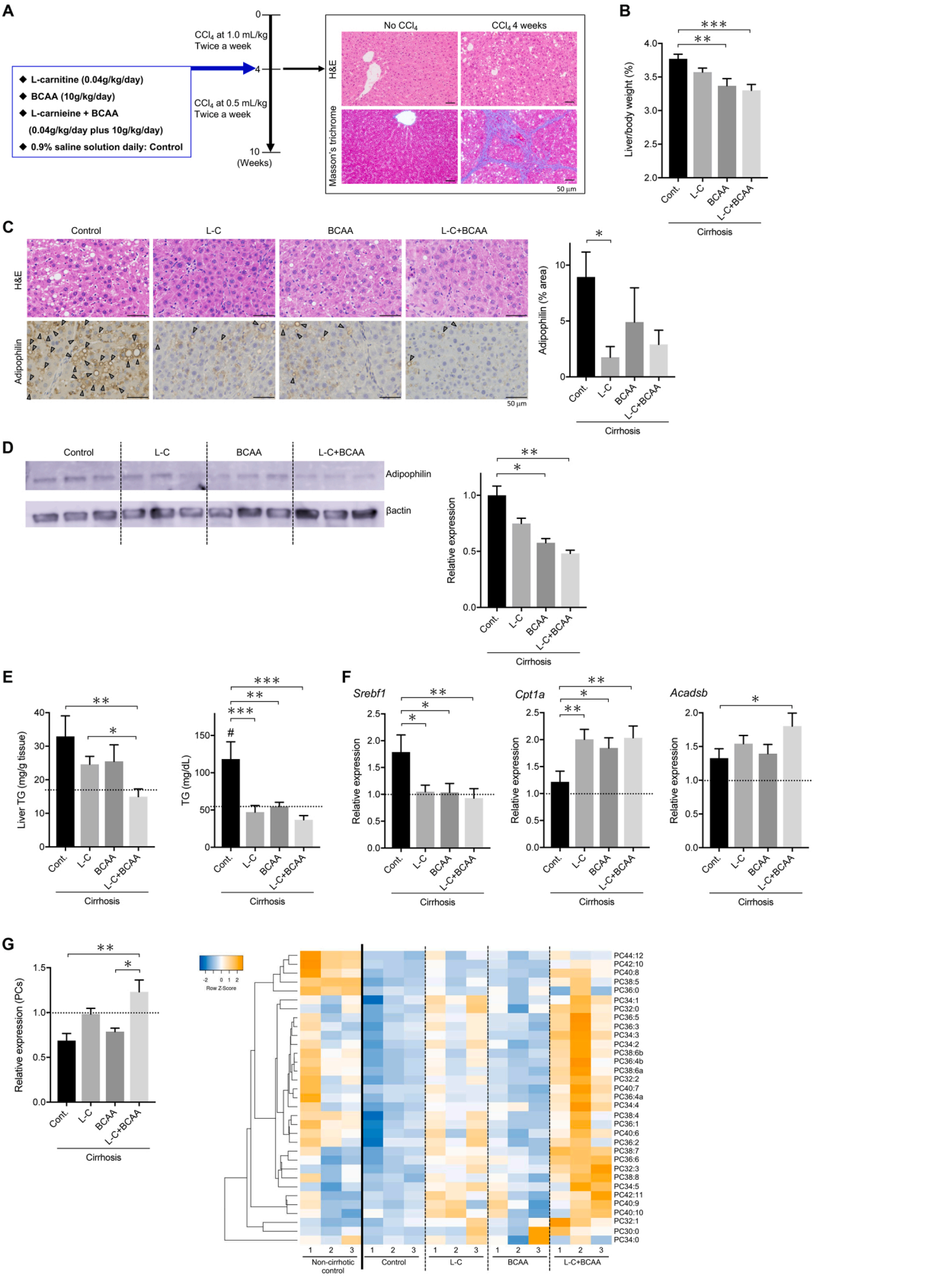
All rats were sacrificed at the conclusion of our treatment protocol under anesthesia (isoflurane, DS-pharma, Osaka, Japan). Whole rat blood was collected and allocated into tubes with or without anticoagulant (EDTA). A portion of liver tissue was fixed in 10 % formalin for 24 h and embedded in paraffin and the remaining liver tissue was flash frozen in liquid nitrogen and stored at -80 °C. TG levels in serum and liver tissue were measured using LabAssay reagent (FUJIFILM Wako Pure Chemical Industrial, Osaka, Japan). The adenosine 5′-triphosphate (ATP) in serum and liver tissue was measured using AMERIC-ATP and AMERIC-ATP (T) kit, respectively (FUJIFILM Wako Pure Chemical) following the manufacturer's instructions.

2.3. Histological analysis and immunohistochemistry

The liver sections were prepared and stained for H&E (hematoxylin and eosin) and Masson's Trichrome stain at a company (Septsapie, Tokyo, Japan). Immunohistochemistry staining for adipophilin (no. 610102, dilution 1:200; PROGEN, Heidelberg, Germany), 4-hydroxynonenal (4-HNE) (no. STA-035, dilution 1:100; Cell Biolabs, San Diego, CA), F4/80 (no. PA5-21399, dilution 1:100, Thermo Fisher Scientific Inc., Carlsbad, CA), and cleaved caspase 3 (no. 9661, dilution 1:200, Cell Signaling Technology, Danvers, MA) was performed in paraffin embedded sections according to the manufacturer's instruction. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed in paraffin embedded tissue using an ApopTag peroxidase in situ apoptosis detection kit (Millipore-Sigma, Burlington, MA). All images were taken by KEENC BZ-X710 (KEYENCE, Osaka, Japan) and quantitated using Image J software (NIH Image).

2.4. Gene expression

Total RNA was isolated from liver tissue using TRI Reagent (Molecular Research Center, Cincinnati, OH) followed by DNase treatment



(caption on next page)

Fig. 1. l-carnitine and BCAA attenuate liver steatosis within the cirrhotic liver. (A) Schematic diagram of treatment administration with Hematoxylin & Eosin (H&E) staining and Mason's trichrome staining in liver sections from rats without CCl_4 (no CCl_4) and with 4-weeks CCl_4 treatment. (B) Changes in liver/body weight in control (Cont.), L-carnitine (L-C), BCAA, and L-C + BCAA treatment groups. (C) H&E and immunohistochemical stainings for adipophilin in liver sections from cirrhotic rats treated with cont., L-C, BCAA, and L-C + BCAA. Scale bar, 50 μm. Arrows show the area of adipophilin positive droplets. Quantification of adipophilin positive area (right panel). (D) Whole liver protein expression of adipophilin from cirrhotic rats treated with cont., L-C, BCAA, and L-C + BCAA using immunoblotting (left panel). Quantification of adipophilin expression by immunoblotting (right panel). The levels were shown relative to control treatment. (E) Liver (left) and serum (right) triglyceride (TG) levels in cont., L-C, BCAA, and L-C + BCAA groups. (F) Gene expression of *Srebf1, Cpt1a,* and *Acadsb* in cirrhotic livers with cont., L-C, BCAA, and L-C + BCAA treatments, as measured by RTqPCR. Gene expression levels were normalized to the housekeeping control, *B2 microglobulin (B2m)*, and shown relative to control expression level. (G) Total liver PC levels (left panel) in cont., L-C, BCAA, and L-C + BCAA groups and heat map of PC species (right panel) in non-cirrhotic, cirrhotic cont., L-C, BCAA, and L-C + BCAA groups measured by LC/MS. Total level of liver PC was shown relative to non-cirrhotic liver expression level. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, # shows a comparison to non-cirrhotic control (# < 0.05). Values are mean ± SEM. The normal baseline of TG, gene expression, and lipid levels in non-cirrhotic rats is shown as broken line.

(Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. The cDNA was synthesized from total RNA using a cDNA Synthesis kit (Takara, Shiga, Japan). Real-time PCR quantification was performed using the KAPA SYBR FAST qPCR master mix (KAPA Biosystems, Wilmington, MA) or a TaqMan gene expression assay (Thermo Fisher Scientific Inc.) for *Sod1*, and the 7300 Real-Time PCR Detection System (Thermo Fisher Scientific Inc.). The PCR primers were used to amplify each gene as listed in Table 1. Mean values of mRNA were normalized to *beta 2 microglobulin (B2m)*.

2.5. Western blotting analysis

Liver was homogenized in RIPA buffer (150 mM NaCl, 1.0 % NP-40, 1 % sodium deoxycholate, 0.1 % sodium dodecyl sulphate, 50 mM Tris-HCl pH8.0) containing a protease inhibitor cocktail (Millipore-Sigma). Mitochondria and cytosolic fractions were isolated with mitochondria isolation kit (Thermo scientific). 20 μg of whole-liver lysate, mitochondria and cytosolic fractions were resolved using a TGX gel (Bio-Rad, Hercules, CA), transferred to a polyvinylidenedifluoride membrane, and blotted with the appropriate primary antibody. Membranes were incubated with peroxidase-conjugated secondary antibody (GE Healthcare Bioscience, Marlborough, MA). Protein bands were visualized using an enhanced chemiluminescence reagent (Bio-Rad), digitized using a Lumino-image analyzer (LAS-4000 iniEPUV, Fuji Film, Tokyo, Japan), and quantitated using the program Multi Gauge (Fuji Film). Anti-adipophilin (no. 610102, dilution 1:2000; PROGEN), anti-4-HNE (no. STA-035, dilution 1:1000; Cell Biolabs), anti-Drp1 (no. 8570, dilution 1:1000, Cell Signaling Technology), anti-βactin (no. 3598R, dilution 1:4000; BioVision, Milpitas, CA), anti-GAPDH (no. 10494, dilution 1:3000, Proteintech, Rosemont, IL), and anti-porin (no. 15895, dilution 1:1000, Abcam, Cambridge, MA) were used as primary antibodies.

Table 1
Oligonucleotides for mRNA expression.

Srebf1	forward	GGAGCCATGGATTGCACATT
	reverse	AGGCCAGGGAAGTCACTGTCT
Cpt1a	forward	CTCCTGAGCAGTTACCAATGC
	reverse	GAACCTTGGCTGCGGTAAGAC
Acadsb	forward	CGATGCGAAGATCGGTACAAT
	reverse	GACGTCAGTACTCTGCATCG
Tfam	forward	GCTTCCAGGAGGCTAAGGAT
	reverse	CCCAATCCCAATGCAACTC
Ndufb8	forward	ACTACGAGCCGTACCCAGAT
	reverse	CCCAGTGTATCGGTTCACCC
F4/80	forward	CTCTTCCTGATGGTGAGAAACC
	reverse	CCCATGGATGTACAGTAGCAGA
Acsl1	forward	AATGATCTGGTGGAACGCGG
	reverse	TTCTGGAGGCTTGGGTTTCG
Bckdhb	forward	CCACGGTCCAGGAAGAATGT
	reverse	GTGAGGAAACGGGGTGTCAT
Sdhd	forward	CAGTCACTGGGGCATTGGAC
	reverse	AGGTCAAAGCTGAGACTGCC
β2m	forward	CGTGATCTTTCTGGTGCTTGTC
	reverse	TTCTGAATGGCAAGCACGAC

2.6. Total lipids extraction

The lipid extraction procedure from liver was performed according to Folch [31]. In brief, the lipid from a liver sample was twice extracted with cold chloroform/methanol 2:1 (*v/v*, with 0.002 % butylated hydroxytoluene (BHT, Sigma-Aldrich, St. Louis, MO) and internal standards (IS) (Avanti Polar Lipids, Alabaster, AL), followed by a combination of extraction and desiccation under vacuum (CC-105, TOMY SEIKO Co., Ltd., Japan). Then, the dried lipids were dissolved in methanol, centrifuged at 680 *g* at 4 °C for 15 min to remove any insoluble material, and stored at −80 °C until analysis. All samples were analyzed within 1 h to avoid lipid degradation and auto-oxidation. All lipid extracts were prepared in triplicate.

2.7. LC/MS analysis

Lipid extracts were separated on an Atlantic T3 C18 column (2.1 × 150 mm, 3 μm, Waters, Milford, MA) connected to a Shimadzu Prominence HPLC system (Shimadzu Corp., Kyoto, Japan). A flow rate of 200 μL/min was used for the analysis, and the column and sample tray were held at 40 °C and 4 °C, respectively. LC gradient elution was performed with a mobile phase of 5 mM aqueous ammonium acetate (Sigma-Aldrich) isopropanol, and methanol (Wako Pure Chemicals). MS analysis was carried out using an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific Inc.). These conditions were held constant for both ESI-positive and ESI-negative ionization mode acquisitions: The MS capillary voltage was set at 3.0 kV, the sheath gas (nitrogen) flow rate was set to 50 units, and the auxiliary gas (nitrogen) flow rate was set to 5 units. The MS data were obtained in Fourier Transform mode with resolving power set to 60,000 and a 2 Hz scan speed, while the tandem MS data were acquired using collision-induced dissociation in ion-trap mode and data-dependent acquisition. The extracted ion chromatograms (EICs) were drawn within the mass tolerance of 5.0 ppm by Xcalibur 2.2 (Thermo Fisher Scientific Inc.). The LC/MS identification of lipid molecules was executed by means of comparison with known standards, along with LipidBlast and an in-house library database [32–34]. The alignment, peak extraction, and EIC peak area integration from the raw data were utilized by the MS-label free differential analysis software package SIEVE 2.0 (Thermo Fisher Scientific Inc.). The amount of each lipid species was calculated as the equation below, as previously reported [35]. Heatmap analysis was conducted with Heatmapper [36] according to the manual.

$$Analyte\ amount = IS\ amount \times \frac{Analyte\ peak\ area}{IS\ peak\ area}$$

2.8. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) from non-cirrhotic control group (n = 3), control group (n = 8), L-carnitine group (n = 9), BCAA group (n = 7), and combination group (n = 8) for all experiments, except western blotting and LC/MS analysis. The mRNA levels were measured with duplication in each rat. For

western blotting and LC/MS analysis, data were expressed as mean ± SEM from control group (n = 3), L-carnitine group (n = 3), BCAA group (n = 3), and combination group (n = 3). Statistical significances were determined using non-parametric Kruskal-Wallis test or using ordinary one-way ANOVA only for factors passed the test of normality. Test of normality was performed using SPSS21.0 software (IBM, Armonk, NY) and all statistical analyses were performed using Prism 7 (GraphPad Software Inc., CA, USA). All tests were two-sided, and $P < 0.05$ was considered significant.

3. Results

3.1. Effect of L-carnitine and BCAA treatments on liver steatosis in the cirrhotic rat model

To investigate the effect of L-carnitine and BCAA treatments on liver cirrhosis, we administered CCl_4 for 4 weeks in order to induce a cirrhotic state within the liver. We observed the increase of steatosis by H&E staining and the regenerating nodules by Masson's trichrome staining in the cirrhotic rats (Fig. 1A). The cirrhotic rats were then treated for 6 weeks via daily gavage with L-carnitine, BCAA, or a combination of L-carnitine plus BCAA (Fig. 1A). Total liver weight was significantly decreased in both the BCAA ($P < 0.01$) and combination ($P < 0.001$) groups (Fig. 1B). H&E staining showed that the deposition of lipid droplets was noticeably decreased in all treatment groups (Fig. 1C). All of lipid droplets were not preserved during the paraffin block preparation due to usage of organic solvents. Therefore, we performed adipophilin, a protein that localizes on the surface of lipid droplets, staining by IHC to examine the presence of lipid droplets. Adipophilin was also decreased in all treatment groups ($P < 0.05$ in L-carnitine) (Fig. 1C). Using quantitative immunoblotting analysis, we also observed a significant decrease of 20–50 % in adipophilin protein levels within whole liver lysates under all treatment groups ($P < 0.05$ in BCAA), particularly in the combination group ($P < 0.01$) (Fig. 1D). Liver and serum TG levels were dramatically increased in the cirrhotic control group, compared with non-cirrhotic rats ($P < 0.05$ in serum TG, Fig. 1E). Corresponding to morphological and histological changes by their treatments in the cirrhotic liver, liver TG levels were decreased in all treatment groups ($P < 0.01$ in combination) with a noticeable combined effect related to the combination group ($P < 0.05$ in L-carnitine vs. combination) (Fig. 1E). In addition, serum TG levels were significantly decreased in all treatment groups as compared with the cirrhotic control group ($P < 0.001$ in L-carnitine and combination, $P < 0.01$ in BCAA) (Fig. 1E). We further examined the expression of genes involved in lipid synthesis, such as *Sterol regulatory element binding transcription factor 1 (Srebf1)* as a key role in the induction of lipogenesis [37], *Carnitine palmitoyltransferase I alpha (Cpt1a)* and *short/branched chain specific acyl-CoA dehydrogenase (Acadsb)* associated with lipolysis and β oxidation [38]. The level of *Srebf1* mRNA was significantly decreased in all treatment groups ($P < 0.05$ in L-carnitine and BCAA, $P < 0.01$ in combination), while mRNA levels of *Cpt1a* and *Acadsb* were increased by L-carnitine and BCAA treatments (*Cpt1a:* $P < 0.01$ in L-carnitine and combination, $P < 0.05$ in BCAA, *Acadsb:* $P < 0.05$ in combination) (Fig. 1F). Liver-specific lipidomic analyses showed total PC and most of 33 PC species, associated with lipid utilization [4], were obviously decreased in the cirrhotic control, compared with non-cirrhotic liver (Fig. 1G). L-carnitine and combination treatments increased total PC ($P < 0.01$ in combination, $P < 0.05$ in BCAA vs. combination) and most PC species in the cirrhotic liver (Fig. 1G). These results demonstrate that L-carnitine and BCAA treatments promote lipid utilization rather than accumulation, which results in an overall reduction of liver steatosis within the cirrhotic liver.

3.2. L-carnitine and BCAA reduce lipid peroxides within the cirrhotic liver

The significant improvement of liver steatosis by treatment with BCAA and L-carnitine led us to explore the hepatic effects of lipid peroxidation. 4-HNE as an end product of lipid peroxidation was abundantly expressed in the control group and showed reduced expression in the L-carnitine, BCAA, and combination groups as assessed by IHC (Fig. 2A) and immunoblotting ($P < 0.01$ in combination, $P < 0.05$ in L-carnitine vs. combination) (Fig. 2B). We next identified the molecular species of lipid peroxide using LC/HR-MS/MS. A total of 11 molecular species of phosphatidylcholine hydroperoxides (PCOOH) and four molecular species of triglyceride hydroperoxides (TGOOH) were accumulated within the cirrhotic liver (Fig. 2C). The heatmap showed that these peroxides, were strongly decreased in the BCAA and combination groups (Fig. 2C). The representative PCOOH36:2, 36:5, 38:4, and 38:6 were significantly increased in cirrhotic control, compared with non-cirrhotic liver ($P < 0.01$ in PCOOH36:2, 36:5, and 38:6; $P < 0.05$ in PCOOH38:4). BCAA and combination treatments decreased these PCOOHs ($P < 0.05$ in PCOOH36:2 and 38:4 BCAA, in PCOOH36:2 combination, and in PCOOH36:5 L-carnitine vs. combination; $P < 0.01$ in PCOOH36:5, 38:4, and 38:6 combination) (Fig. 2D). The TGOOH52:3, 52:4, and 54:5 were also increased in cirrhotic control compared with non-cirrhotic liver ($P < 0.05$ in TGOOH52:3 and 52:4). These TGOOHs were significantly decreased in the BCAA ($P < 0.05$) and combination groups ($P < 0.05$ in TGOOH52:3 and 54:5, in TGOOH 52:4 L-carnitine vs. combination; $P < 0.01$ in TGOOH 52:4) (Fig. 2E). Most of the lipid peroxide species were recovered at non-cirrhotic level (shown as broken line) by L-carnitine and BCAA treatments, especially combination treatment. Paradoxically, the reduction of lipid peroxides was accompanied by the increased mRNA levels of *Superoxide dismutase 1 (Sod1)* as a major hepatic antioxidant enzyme in the combination group ($P < 0.01$, $P < 0.05$ in L-carnitine or BCAA vs. combination) (Fig. 2F). These results reveal that L-carnitine and BCAA treatments reduce lipid peroxidation within the cirrhotic liver.

3.3. L-carnitine and BCAA maintain mitochondrial function within the cirrhotic liver

The overall improvement of liver steatosis and the reduction of lipid peroxides indicate the recovery of mitochondrial function influenced by L-carnitine and BCAA treatments. Cardiolipins (CLs) including CL68:2−6, CL70:4−7, CL72:6−9, CL74:7−11, and CL76:9−12 are an essential component of the mitochondrial inner-membrane and directly interact with respiratory chain proteins (Fig. 3A). Total CLs were significantly decreased in cirrhotic control compared with non-cirrhotic liver ($P < 0.01$) and were increased by L-carnitine and BCAA treatments ($P < 0.05$ in BCAA) (Fig. 3B). Nascent CLs considered as precursors in CL biosynthesis, such as CL68 and CL70, were not changed in all treatment groups (data not shown). In contrast, functional CLs, such as CL72, CL74, and CL76, were decreased in the cirrhotic control compared with non-cirrhotic liver ($P < 0.05$ in CL74:7, 74:9, and 76:9; $P < 0.01$ in CL72:8 and 74:8) and tended to elevate in all treatment groups (Fig. 3C). Especially, CL72:8, 74:9, and 76:10 were significantly increased in the BCAA group ($P < 0.05$ in all), whereas CL74:7, 74:9, and 76:9 were significantly increased in the combination group ($P < 0.05$ in CL74:4 and 74:9; $P < 0.01$ in CL76:9) (Fig. 3C). In addition, L-carnitine and BCAA treatments recovered the amount of mature CL74 and 76 to/above the normal level (shown as broken line), resulting in the acceleration of CL remodeling within the L-carnitine and BCAA groups. We observed that liver mRNA levels of the mitochondrial biogenesis gene *Mitochondrial transcription factor A (Tfam)* were dramatically decreased in cirrhotic control compared with non-cirrhotic liver ($P < 0.0001$) and were increased in combination group ($P < 0.001$ in combination, $P < 0.01$ in L-carnitine vs. combination, $P < 0.05$ BCAA vs. combination), although it was not recovered to the same level of non-cirrhotic liver ($P < 0.0001$ in treatment groups vs. non-cirrhotic liver) (Fig. 3D). Liver mRNA levels of *NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 8 (Ndufb8)* as the mitochondrial respiratory complex marker were tended toward an increase in both the L-carnitine and combination groups (Fig. 3D). We further examined the formation

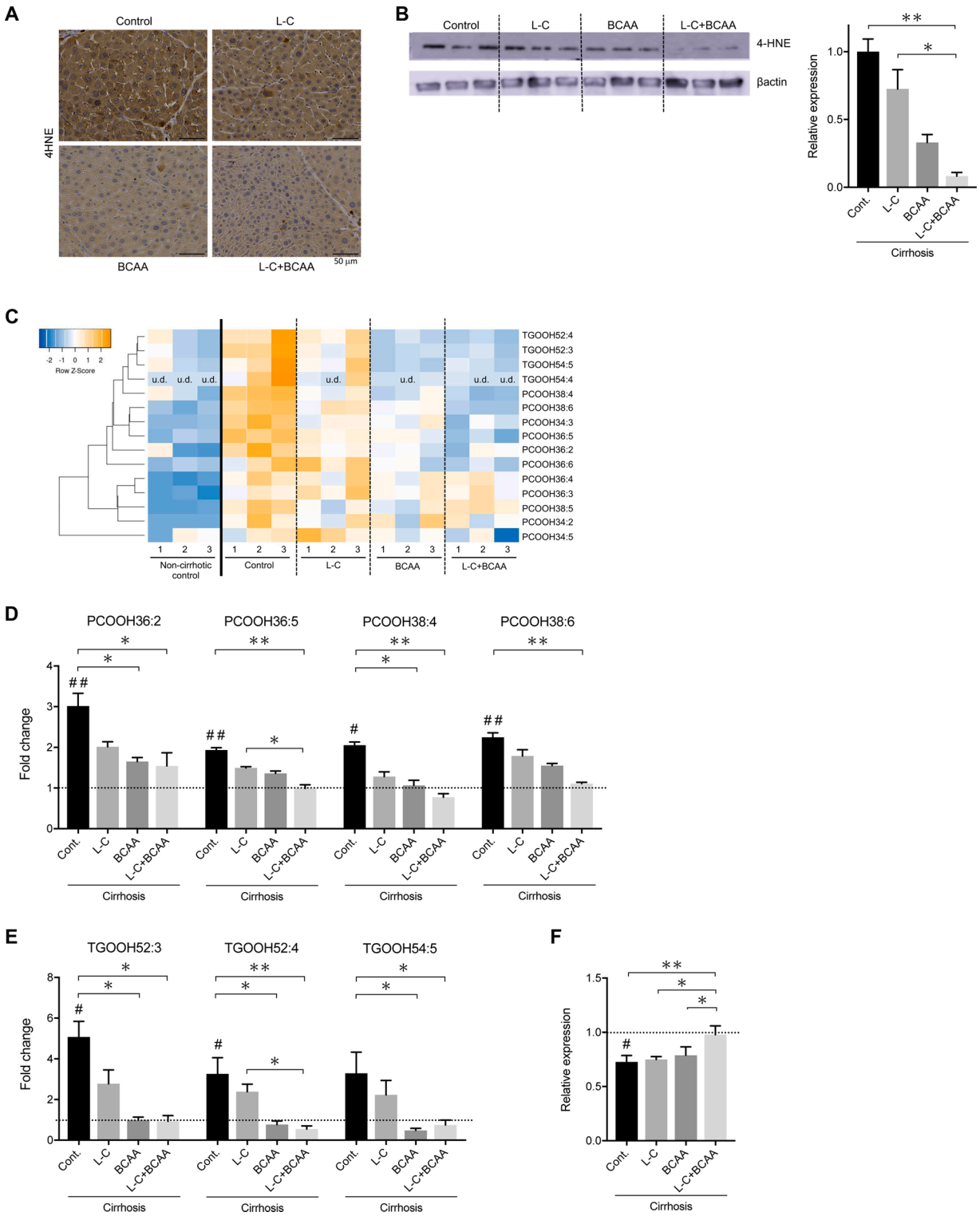


Fig. 2. Effect of l-carnitine and BCAA treatments on lipid peroxidation in the cirrhotic liver. (A) Immunohistochemical staining for 4-HNE in liver sections from cirrhotic rats treated with control (Cont.), L-carnitine (L-C), BCAA, and L-C + BCAA. Scale bar, 50 μm. (B) Whole liver protein expression of 4-HNE from cirrhotic rats given L-C and BCAA treatments using immunoblotting (left panel). Bar graph shows quantification of 4-HNE from immunoblotting (right panel). (C) Heat map of 11 phosphatidylcholine hydroperoxides (PCOOH) and 4 triacylglycerol hydroperoxides (TGOOH) in livers with non-cirrhotic, cirrhotic cont., L-C, BCAA, and L-C + BCAA treatments. u.d.; undetectable. (D and E) Comparison of representative liver lipid peroxides of PCOOH36:2, 36:5, 38:4, and 38:6 (D), or TGOOH52:3, 52:4, and 54:5 (E) in cirrhotic livers with cont., L-C, BCAA, and L-C + BCAA treatments measured by LC/HR-MS/MS. (F) Gene expression of *Sod1* in cirrhotic livers with cont., L-C, BCAA, and L-C + BCAA treatments, as measured by RTqPCR. * $P < 0.05$, ** $P < 0.01$, # shows a comparison to non-cirrhotic liver (# < 0.05, ## < 0.01). Values are mean ± SEM. The normal baseline of lipid peroxides and *Sod1* gene expression in non-cirrhotic rats is shown as broken line.

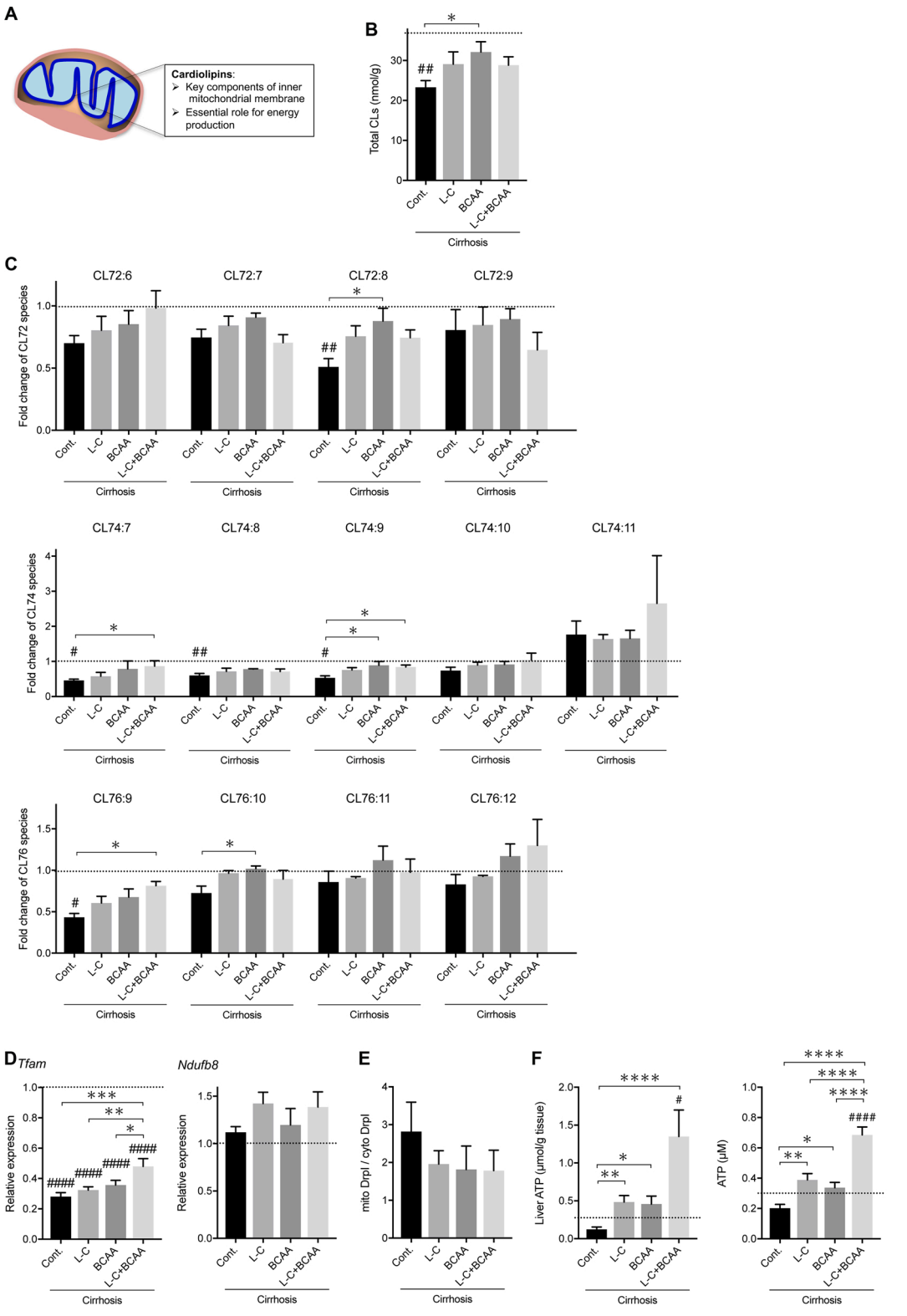


Fig. 3. L-carnitine and BCAA treatments recover mitochondrial integrity in the cirrhotic liver. (A) Illustration of cardiolipin (CL) function in mitochondrion. (B) Total CLs levels in cirrhotic livers with control (Cont.), L-carnitine (L-C), BCAA, and L-C + BCAA treatments. (C) Representative functional CLs levels in cirrhotic livers with cont., L-C, BCAA, and L-C + BCAA treatments measured by LC/MS. (D) Gene expression of *Tfam* and *Ndufb8* in cirrhotic livers with cont., L-C, BCAA, and L-C + BCAA treatments, as measured by RTqPCR. (E) Localization of Drp1 in mitochondria (mito) and cytosolic (cyto) fractions isolated from cirrhotic livers with cont., L-C, BCAA, and L-C + BCAA treatments. The mito Drp1 levels were shown as relative to the cyto Drp1 level, determined by quantitative immunoblotting. (F) Liver (left) and serum (right) ATP levels in cirrhotic rats with cont., L-C, BCAA, and L-C + BCAA treatments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, # shows a comparison to non-cirrhotic liver (# < 0.05, ## < 0.01, #### < 0.0001). Values are mean ± SEM. The normal baseline of gene expression, ATP, lipid composition, and cardiolipins in non-cirrhotic rats is shown as broken line.

of mitochondria by immunoblotting using anti-dynamin-related protein 1 (Drp1) antibody. Drp1 is a fundamental component of mitochondrial fission and its binding induces the breakdown of damaged mitochondria [39]. We compared the Drp1 localisation between the mitochondria (mito) and cytosolic (cyto) fractions isolated from their livers (suppl. Fig. 1A). Mitochondria Drp1 was relatively decreased in all treatment groups (Fig. 3E), suggesting both BCAA and L-carnitine promote to regenerate functional mitochondria within the cirrhotic liver. These results clearly support our hypothesis that L-carnitine and BCAA treatments recover mitochondrial function within the cirrhotic liver. To confirm this, we examined overall ATP production in the liver and in circulation via the serum. The total level of ATP within the liver was significantly elevated in all treatment groups when compared with the control group ($P < 0.01$ in L-carnitine; $P < 0.05$ in BCAA, in combination vs. non-cirrhotic liver, $P < 0.0001$ in combination) (Fig. 3E). Furthermore, serum ATP levels were significantly increased in all treatment groups ($P < 0.01$ in L-carnitine; $P < 0.05$ in BCAA; $P < 0.0001$ in combination, in combination vs. non-cirrhotic liver) with combined effects in the combination group ($P < 0.0001$ in L-carnitine or BCAA vs. combination) (Fig. 3E). These results suggest that L-carnitine and BCAA treatments reorganize mitochondrial structure and function within the cirrhotic liver.

3.4. L-carnitine and BCAA attenuate hepatocyte damages within the cirrhotic liver

From the improvement of liver steatosis, reduction of lipid peroxides, and recovery of mitochondrial integrity, we expect that L-carnitine and BCAA treatments attenuate hepatocyte damages in cirrhotic rats. Firstly, we examined the liver fibrosis by trichrome staining. There was no significant change by their treatments for 6 weeks (Fig. 4A), although long-term (16 weeks) BCAA treatment clearly attenuated the liver fibrosis in cirrhotic rats [25]. Next we assessed liver inflammation using F4/80 staining and mRNA expression. L-carnitine and BCAA treatments did not lead to significant changes of the number and level of F4/80 in the cirrhotic liver (Fig. 4B). There results suggest that short-term L-carnitine and BCAA treatments did not lead to a significant reduction of liver fibrosis and inflammation in the cirrhotic liver. We also measured lipotoxic intermediates from lipidomic analysis. Whereas the ratio of PC/PE which is as index of liver health was significantly below 1.0 in the cirrhotic livers ($P < 0.05$ vs. non-cirrhotic liver), it was recovered in all treatment groups, especially in the combination group with almost 1.5 ($P < 0.05$) (Fig. 4C). Furthermore, total and most of LysoPE species which are as index of cellular protection were increased within the BCAA and combination groups ($P < 0.05$ in combination) (Fig. 4D). Lastly, we examined liver apoptosis using TUNEL and cleaved caspase 3 staining. Although the number of TUNEL-positive and cleaved caspase 3-positive hepatocytes were increased in the cirrhotic liver, they were significantly decreased in all treatment groups (TUNEL: $P < 0.01$ in L-carnitine; $P < 0.001$ in BCAA and combination, cleaved caspase 3: $P < 0.001$ in all groups) (Fig. 4E and F). These results suggest that L-carnitine and BCAA treatments reduce hepatocellular damage by lipotoxicity within the cirrhotic liver.

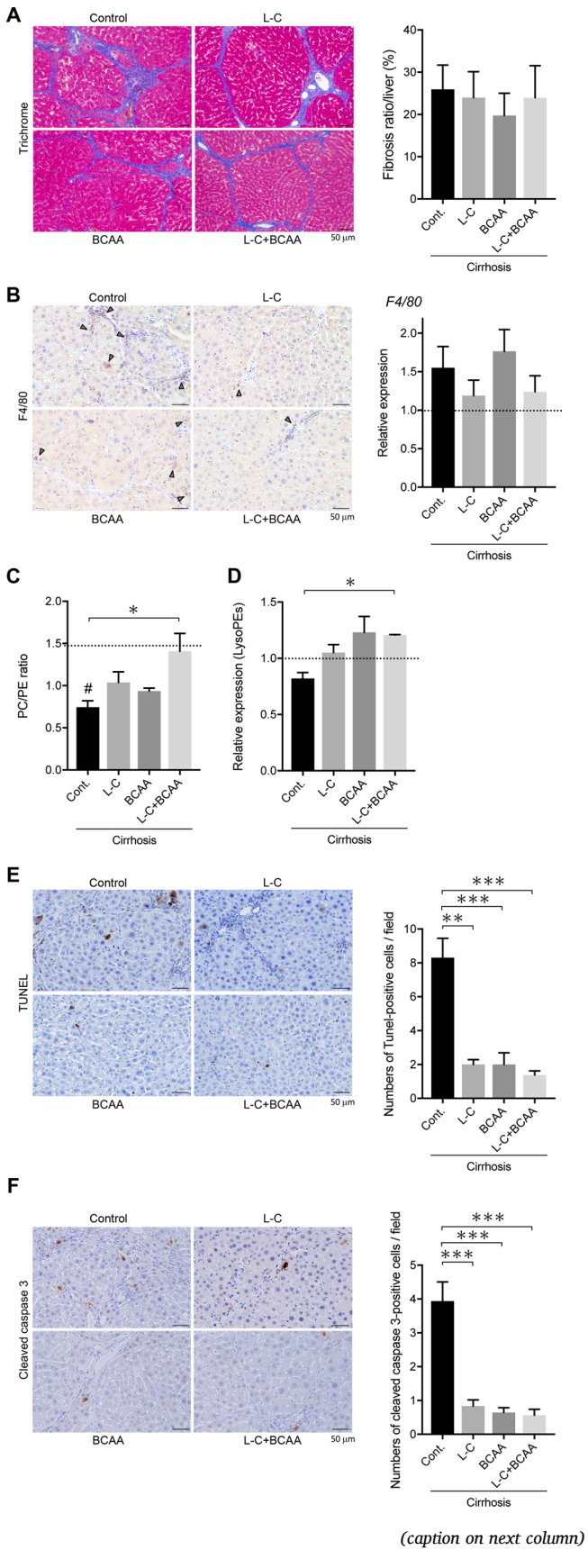
As a conclusion, these results suggest that L-carnitine and BCAA potentiate the remodeling of CLs and support mitochondrial function leading to a reduction in hepatocellular damage. Based on all presented data, cirrhotic hepatocytes have lipid deposition, increased lipid peroxides (TGOOH, PCOOH) and reduced mitochondrial function resulting in increased hepatocyte cell death (Fig. 5, left panel). L-carnitine and BCAA promote lipolysis and glycogen synthesis via the replenishment of Acyl-CoA and a reactivation of the TCA cycle. Our results show that a treatment profile of L-carnitine and BCAA can protect mitochondrial integrity via subsequent recovery of CLs, as well as increase ATP production thus attenuating lipid peroxide production resulting in the overall protection of the cirrhotic liver (Fig. 5, right panel).

4. Discussion

This study has demonstrated the efficacy of oral administration of L-carnitine and BCAA to attenuate lipotoxic hepatocellular damage in cirrhotic rats through the reduction of lipid peroxides, the recovery of mitochondrial function, and a diminution of hepatocyte death.

One distinctive feature of the CCl_4-induced animal model is a rapid accumulation of lipid within the cirrhotic liver [2,40], which mimics the phenotype of lipid accumulation (steatosis) in human cirrhotic patients [2]. Indeed, we clearly observed an increase in liver steatosis within the livers of our CCl_4-induced cirrhotic rat model, as evidenced by the adipophilin IHC staining. Notably, liver steatosis was significantly decreased in cirrhotic rats treated with L-carnitine, BCAA, and combination. Moreover, the reduction in liver steatosis was accompanied by a decrease in both liver and serum TG levels upon treatment with L-carnitine, BCAA, and combination. In conjunction, we observed an increase in phospholipid integrity, which portends more robust membrane constitution and fusion, and the availability of important precursors related to phosphoglycerolipid metabolic pathways. These results indicate a potential for L-carnitine and BCAA to accelerate the hydrolyzing of TG from hepatocyte lipid droplets and reconstructing them to other lipid classes. Therefore, L-carnitine and BCAA improve the overall lipid profile and fat accumulation within the liver, and then attenuate liver steatosis in our cirrhotic model. However, both BCAA and L-carnitine are similar effect in liver steatosis, we have demonstrated first time that L-carnitine specifically activated PC synthesis in the cirrhotic liver. This is a distinguishable efficacy from BCAA treatment. Liver steatosis is also associated with insulin resistance as a result of an increase in hepatic glucose production via the up-regulation of gluconeogenesis [41,42]. Indeed, the increased serum insulin levels and homeostasis model assessment for insulin resistance (HOMA-R) seen in cirrhotic rats were dramatically attenuated as a result of treatment with L-carnitine, BCAA, and combination (data not shown), resulting in an overall improvement of glucose metabolism associated with a reduction in liver steatosis. We observed that there were no significant changes related to serum FFA and total cholesterol levels within all treatment groups (data not shown). We further examined the mRNA expression profiles associated with lipid metabolism because BCAA and L-carnitine are involved in lipid metabolism by means of Acyl-CoA production and the TCA cycle (suppl. Fig. 2A). In Fig. 1F, we observed mRNA levels of *Srebf1, Cpt1a* and *Acadsb* were significantly changed by L-carnitine and/or BCAA treatments. The mRNA level of *Acsl1* as a key enzyme of fatty acid metabolism and *Bckdhb* as a component of the BCKDH complex were not significantly changed in all treatment groups (suppl. Fig. 2B). The level of *Sdhd* mRNA as a component of TCA cycle was increased in all treatment groups ($P < 0.05$ in BCAA) (suppl. Fig. 2B). These results indicate that both BCAA and L-carnitine indirectly affect fatty acid metabolism within the cirrhotic liver. Lipid peroxidation has been linked to inflammation, carcinogenesis and aging [43,44]. The concentration of PCOOH, a primary lipid peroxidation product of LDL or VLDL, has been shown to be correlated with the amount of circulating hydrogen peroxide and oxidized LDL [45]. Therefore, the semi-quantitative analysis of PCOOH is a critical step in the evaluation of the biochemical processes leading to oxidative injury. Indeed, PCOOH levels in plasma were increased in alcoholic patients due to increased oxidative stress [46], but the measurement of lipid peroxides proved to be difficult due to the low concentration of lipid peroxides in serum [47]. In this study, we detected 11 molecular species of PCOOH and four species of TGOOH in the cirrhotic liver, by which we used to address and compare the direct amount of lipid peroxides in the liver. The most of TGOOH and PCOOH molecular species was significantly increased in the cirrhotic liver and was significantly decreased by BCAA treatment, with the most pronounced effect seen in the group receiving the combination therapy. These results suggest that BCAA has more anti-lipid peroxidation activity than L-carnitine in the cirrhotic liver.

The amount of CL molecular species is an important index in



(caption on next column)

Fig. 4. l-carnitine and BCAA treatments protect hepatocellular damage in cirrhotic liver. (A) Masson's trichrome staining in liver sections from cirrhotic rats treated with control (Cont.), L-carnitine (L-C), BCAA, and L-C + BCAA treatments (left panel). Scale bar, 50 μm. Quantification of trichrome positive area (right panel). (B) Immunohistochemical staining for F4/80 in liver sections from cirrhotic rats treated with cont., L-C, BCAA, and L-C + BCAA (left panel). Scale bar, 50 μm. Arrows show the area of F4/80 expression. Gene expression of *F4/80* in cirrhotic livers with cont., L-C, BCAA, and L-C + BCAA treatments, as measured by RTqPCR (right panel). (C) Liver lipid PC/PE ratios in cont., L-C, BCAA, and L-C + BCAA groups measured by LC/MS. (D) Total liver LysoPE levels in cont., L-C, BCAA, and L-C + BCAA groups measured by LC/MS and shown relative to non-cirrhotic liver level. (E) TUNEL stainings of liver sections in cirrhotic rats treated with cont., L-C, BCAA, and L-C + BCAA (left panel). Scale bar, 50 μm. Quantification of TUNEL positive cells (right panel). (F) Immunohistochemical staining for cleaved caspase 3 in liver sections from cirrhotic rats treated with cont., L-C, BCAA, and L-C + BCAA (left panel). Scale bar, 50 μm. Quantification of cleaved caspase 3 positive cells (right panel). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, # shows a comparison to non-cirrhotic control (# < 0.05). Values are mean ± SEM. The normal baseline of gene expression and lipid composition in non-cirrhotic rats is shown as broken line.

recognizing the overall mitochondrial structural integrity and associated electron transport system [48,49]. CLs are glycerol-bridged dimericphospholipids and are essential components of mitochondrial cristae formation. Therefore, CLs directly interact with respiratory chain proteins, including complex I (ubiquinone oxidoreductase), complex III (cytochrome c oxidoreductase), complex IV (cytochrome c oxidase), and complex V (ATP synthase), as well as adenine nucleotide translocator, phosphate carrier, carnitine palmitoyltransferase, mitochondrial creatine kinase, and mitochondrialnucleoside diphosphate kinase [49–51]. We found that the amounts of total and mature CLs (CL72, 74 and 76) were predominantly increased in all treatment groups. Furthermore, L-carnitine and BCAA treatments also relatively increased the expression of the genes implicated with mitochondrial function such as *Cpt1a, Acadsb* (Fig. 1F), *Tfam, Ndufb8* (Fig. 3D) and *Sdhd* (suppl. Fig. 2B). Consequently, liver and serum ATP productions were significantly increased in the combination treatment group. These results display combined effects of L-carnitine and BCAA in mitochondrial structure and function. However, further analyses are warranted to better understand the combined efficacy in mitochondrial function using fresh liver samples, a combination treatment protocol would be the option for patients presenting with liver cirrhosis.

L-carnitine and BCAA as well as dietary supplementation could improve fatty acid catabolism and oxidation [52–54]. The suppression of lipotoxicity by L-carnitine and BCAA led to a significant protection of hepatocellular damage, but not yet to reach the significant changes of liver fibrosis and inflammation, as well as serum albumin and alanine transferase levels (data not shown). We had reported that BCAA treatment within 16 weeks attenuated the liver fibrosis in cirrhotic rats [25], suggesting that longer term of L-carnitine and BCAA treatments would require to overcome liver fibrosis and inflammation. Furthermore, current studies revealed that extracellular vesicles from damaged hepatocytes activated hepatic stellate cells (HSCs) and hepatic macrophages (HMs) [55], thus a significant protection of hepatocellular damage by L-carnitine and BCAA treatments in this study will contribute to inhibit the activation loop from the damaged hepatocytes to HSCs and HMs. Future study should examine whether L-carnitine and BCAA affect the activation of these cells.

Author contributions

Y.T. performed most of the experiments, analyzed the data, and wrote the manuscript. Z.C., Y.W., H.C., and S.P. contributed to lipid experiments. M.I. and A.E. designed most of the experiments and analyzed the data. J.O., Y.K., M.I., M.I., and Y.T. participated in the design, execution and analysis of the study. All authors have discussed results from the experiments, read and approved the final version of the

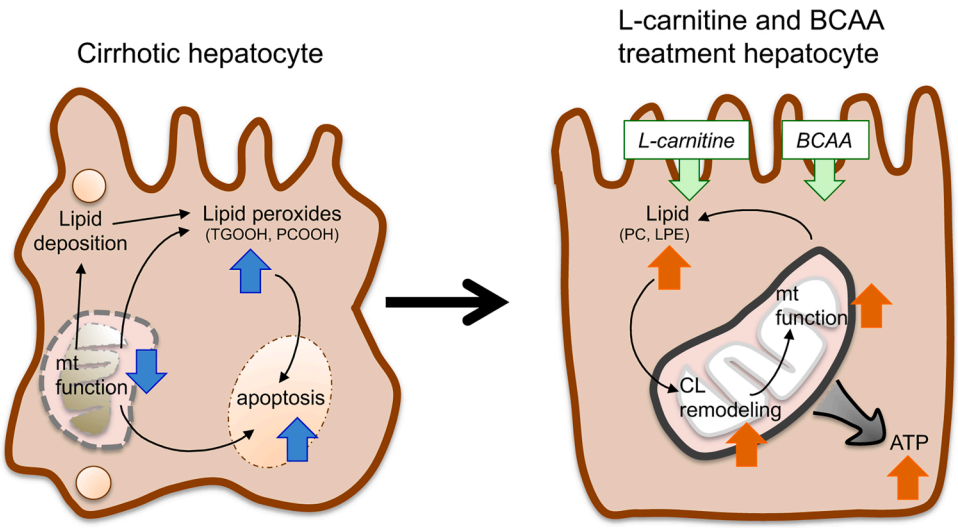


Fig. 5. Putative role of BCAA and l-carnitine treatments in cirrhotic liver. A schematic illustration of the role of L-carnitine and BCAA treatments in the cirrhotic hepatocyte. There was a negative cycle of increased lipid peroxides, mitochondrial dysfunction and apoptosis in cirrhotic hepatocytes (left panel). L-carnitine and BCAA led to a positive cycle with increased lipid synthesis and CL remodeling, recovery of mitochondrial integrity, and an increase in ATP production resulting in a reversal of hepatocellular damage within the cirrhotic liver (right panel).

submitted manuscript.

Funding statement

No external financial support was received from funding agencies in the pubic, except maintaining cirrhotic rats from Otsuka Pharmaceutical Company.

Ethics approval statement

Our animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Hokudo Co., Ltd (Sapporo, Japan).

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

We thank Mrs. Mina Tempaku (Department of Gastroenterology and Hepatology, Mie University Graduate School of Medicine) for her technical assistance.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.111181.

References

[1] S.K. Natarajan, S. Thomas, P. Ramamoorthy, J. Basivireddy, A.B. Pulimood, A. Ramachandran, K.A. Balasubramanian, Oxidative stress in the development of liver cirrhosis: a comparison of two different experimental models, J. Gastroenterol. Hepatol. 21 (6) (2006) 947–957.

[2] X. Pan, F.N. Hussain, J. Iqbal, M.H. Feuerman, M.M. Hussain, Inhibiting proteasomal degradation of microsomal triglyceride transfer protein prevents CCl4-induced steatosis, J. Biol. Chem. 282 (23) (2007) 17078–17089.

[3] S. Kawasaki, H. Imamura, Y. Bandai, K. Sanjo, Y. Idezuki, Direct evidence for the intact hepatocyte theory in patients with liver cirrhosis, Gastroenterology 102 (4 Pt 1) (1992) 1351–1355.

[4] J.N. van der Veen, J.P. Kennelly, S. Wan, J.E. Vance, D.E. Vance, R.L. Jacobs, The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease, Biochim. Biophys. Acta Biomembr. 1859 (9 Pt B) (2017) 1558–1572.

[5] H. Ono, H. Shimano, H. Katagiri, N. Yahagi, H. Sakoda, Y. Onishi, M. Anai, T. Ogihara, M. Fujishiro, A.Y. Viana, Y. Fukushima, M. Abe, N. Shojima, M. Kikuchi, N. Yamada, Y. Oka, T. Asano, Hepatic Akt activation induces marked hypoglycemia, hepatomegaly, and hypertriglyceridemia with sterol regulatory element binding protein involvement, Diabetes 52 (12) (2003) 2905–2913.

[6] R.L. Jacobs, C. Devlin, I. Tabas, D.E. Vance, Targeted deletion of hepatic CTP: phosphocholine cytidylyltransferase alpha in mice decreases plasma high density and very low density lipoproteins, J. Biol. Chem. 279 (45) (2004) 47402–47410.

[7] A. Nishina, H. Kimura, A. Sekiguchi, R.H. Fukumoto, S. Nakajima, S. Furukawa, Lysophosphatidylethanolamine in Grifola frondosa as a neurotrophic activator via activation of MAPK, J. Lipid Res. 47 (7) (2006) 1434–1443.

[8] A. Pathil, A. Warth, W. Chamulitrat, W. Stremmel, The synthetic bile acid-phospholipid conjugate ursodeoxycholyl lysophosphatidylethanolamide suppresses TNFalpha-induced liver injury, J. Hepatol. 54 (4) (2011) 674–684.

[9] S. Schuster, C.D. Johnson, M. Hennebelle, T. Holtmann, A.Y. Taha, I.A. Kirpich, A. Eguchi, C.E. Ramsden, B.G. Papouchado, C.J. McClain, A.E. Feldstein, Oxidized linoleic acid metabolites induce liver mitochondrial dysfunction, apoptosis, and NLRP3 activation in mice, J. Lipid Res. 59 (9) (2018) 1597–1609.

[10] S.S. Sundaram, A. Halbower, Z. Pan, K. Robbins, K.E. Capocelli, J. Klawitter, C. T. Shearn, R.J. Sokol, Nocturnal hypoxia-induced oxidative stress promotes progression of pediatric non-alcoholic fatty liver disease, J. Hepatol. 65 (3) (2016) 560–569.

[11] Y. Sasaki, A. Dehnad, S. Fish, A. Sato, J. Jiang, J. Tian, K. Schroder, R. Brandes, N. J. Torok, NOX4 regulates CCR2 and CCL2 mRNA stability in alcoholic liver disease, Sci. Rep. 7 (2017) 46144.

[12] T. Sakurai, A. Ichikawa, H. Furukawa, N. Wada, A. Nagasaka, Y. Takahashi, M. Fujikawa, A. Ikuta, H. Furumaki, M. Shiga, C. Shimizu, S.P. Hui, S. Jin, S. Takeda, H. Fuda, H. Nagasaka, S. Kobayashi, H. Chiba, Novel monoclonal antibody recognizing triglyceride-rich oxidized LDLs associated with severe liver disease and small oxidized LDLs in normal subjects, Ann. Clin. Biochem. 49 (5) (2012) 456–462.

[13] S. Hui, T. Murai, T. Yoshimura, H. Chiba, H. Nagasaka, T. Kurosawa, Improved HPLC assay for lipid peroxides in human plasma using the internal standard of hydroperoxide, Lipids 40 (2005) 515.

[14] EASL Clinical Practice Guidelines on nutrition in chronic liver disease, J. Hepatol. 70 (1) (2019) 172–193.

[15] P. Tandon, M. Raman, M. Mourtzakis, M. Merli, A practical approach to nutritional screening and assessment in cirrhosis, Hepatology 65 (3) (2017) 1044–1057.

[16] S. Dasarathy, M. Merli, Sarcopenia from mechanism to diagnosis and treatment in liver disease, J. Hepatol. 65 (6) (2016) 1232–1244.

[17] M. Merli, O. Riggio, L. Dally, Does malnutrition affect survival in cirrhosis? PINC (Policentrica Italiana Nutrizione Cirrosi), Hepatology 23 (1996) 1041–1046.

[18] B. Reuter, J. Shaw, J. Hanson, V. Tate, C. Acharya, J.S. Bajaj, Nutritional assessment in inpatients with cirrhosis can be improved after training and is associated with lower readmissions, Liver Transpl. (2019).

[19] C.J. Chen, L.C. Wang, H.T. Kuo, Y.C. Fang, H.F. Lee, Significant effects of late evening snack on liver functions in patients with liver cirrhosis: a meta-analysis of randomized controlled trials, J. Gastroenterol. Hepatol. 34 (7) (2019) 1143–1152.

[20] M. Ohara, K. Ogawa, G. Suda, M. Kimura, O. Maehara, T. Shimazaki, K. Suzuki, A. Nakamura, M. Umemura, T. Izumi, N. Kawagishi, M. Nakai, T. Sho, M. Natsuizaka, K. Morikawa, S. Ohnishi, N. Sakamoto, L-carnitine suppresses loss of skeletal muscle mass in patients with liver cirrhosis, Hepatol. Commun. 2 (8) (2018) 906–918.

[21] G. Marchesini, G. Bianchi, M. Merli, P. Amodio, C. Panella, C. Loguercio, F. Rossi Fanelli, R. Abbiati, Nutritional supplementation with branched-chain amino acids in advanced cirrhosis: a double-blind, randomized trial, Gastroenterology 124 (7) (2003) 1792–1801.

[22] T. Kawaguchi, N. Izumi, M.R. Charlton, M. Sata, Branched-chain amino acids as pharmacological nutrients in chronic liver disease, Hepatology 54 (3) (2011) 1063–1070.

[23] H. Nishikawa, M. Shiraki, A. Hiramatsu, K. Moriya, K. Hino, S. Nishiguchi, Japan Society of Hepatology guidelines for sarcopenia in liver disease (1st edition):

recommendation from the working group for creation of sarcopenia assessment criteria, Hepatol. Res. 46 (10) (2016) 951–963.
[24] H. Hidaka, T. Nakazawa, S. Kutsukake, Y. Yamazaki, I. Aoki, S. Nakano, N. Asaba, T. Minamino, J. Takada, Y. Tanaka, Y. Okuwaki, M. Watanabe, A. Shibuya, W. Koizumi, The efficacy of nocturnal administration of branched-chain amino acid granules to improve quality of life in patients with cirrhosis, J. Gastroenterol. 48 (2) (2013) 269–276.
[25] M. Iwasa, Y. Kobayashi, R. Mifuji-Moroka, N. Hara, H. Miyachi, R. Sugimoto, H. Tanaka, N. Fujita, E.C. Gabazza, Y. Takei, Branched-chain amino acid supplementation reduces oxidative stress and prolongs survival in rats with advanced liver cirrhosis, PLoS One 8 (7) (2013), e70309.
[26] V. Tanphaichitr, P. Leelahagul, Carnitine metabolism and human carnitine deficiency, Nutrition 9 (3) (1993) 246–254.
[27] S. Krahenbuhl, Carnitine metabolism in chronic liver disease, Life Sci. 59 (19) (1996) 1579–1599.
[28] Q. Jiang, G. Jiang, K.Q. Shi, H. Cai, Y.X. Wang, M.H. Zheng, Oral acetyl-L-carnitine treatment in hepatic encephalopathy: view of evidence-based medicine, Ann. Hepatol. 12 (5) (2013) 803–809.
[29] H. Vidot, S. Carey, M. Allman-Farinelli, N. Shackel, Systematic review: the treatment of muscle cramps in patients with cirrhosis, Aliment. Pharmacol. Ther. 40 (3) (2014) 221–232.
[30] M. Iwasa, R. Sugimoto, T. Ishihara, N. Sekoguchi-Fujikawa, K. Yoshikawa, R. Mifuji-Moroka, H. Tanaka, Y. Kobayashi, H. Hasegawa, Y. Takei, Usefulness of Levocarnitine and/or Branched-Chain Amino Acids during Invasive Treatment for Hepatocellular Carcinoma, J. Nutr. Sci. Vitaminol. (Tokyo) 61 (6) (2015) 433–440.
[31] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[32] S.P. Hui, H. Chiba, S. Jin, H. Nagasaka, T. Kurosawa, Analyses for phosphatidylcholine hydroperoxides by LC/MS, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 878 (20) (2010) 1677–1682.
[33] Z. Chen, Y. Wu, Y.S. Ma, Y. Kobayashi, Y.Y. Zhao, Y. Miura, H. Chiba, S.P. Hui, Profiling of cardiolipins and their hydroperoxides in HepG2 cells by LC/MS, Anal. Bioanal. Chem. 409 (24) (2017) 5735–5745.
[34] T. Kind, K.H. Liu, D.Y. Lee, B. DeFelice, J.K. Meissen, O. Fiehn, LipidBlast in silico tandem mass spectrometry database for lipid identification, Nat. Methods 10 (2014) 755–758.
[35] Z. Chen, Q. Liang, Y. Wu, Z. Gao, S. Kobayashi, J. Patel, C. Li, F. Cai, Y. Zhang, C. Liang, H. Chiba, S.P. Hui, Comprehensive lipidomic profiling in serum and multiple tissues from a mouse model of diabetes, Metabolomics 16 (11) (2020) 115.
[36] S. Babicki, D. Arndt, A. Marcu, Y. Liang, J.R. Grant, A. Maciejewski, D.S. Wishart, Heatmapper: web-enabled heat mapping for all, Nucleic Acids Res. 44 (W1) (2016) W147–53.
[37] H. Shimano, R. Sato, SREBP-regulated lipid metabolism: convergent physiology - divergent pathophysiology, Nat. Rev. Endocrinol. 13 (12) (2017) 710–730.
[38] T.A. Shaw, R. Singaravelu, M.H. Powdrill, J. Nhan, N. Ahmed, D. Ozcelik, J. P. Pezacki, MicroRNA-124 regulates fatty acid and triglyceride homeostasis, iScience 10 (2018) 149–157.
[39] E. Smirnova, D.L. Shurland, S.N. Ryazantsev, A.M. van der Bliek, A human dynamin-related protein controls the distribution of mitochondria, J. Cell Biol. 143 (2) (1998) 351–358.
[40] T.H. Tseng, W.L. Lin, C.K. Chang, K.C. Lee, S.Y. Tung, H.C. Kuo, Protective effects of Morus root extract (MRE) against lipopolysaccharide-activated RAW264.7 cells and CCl4-induced mouse hepatic damage, Cell. Physiol. Biochem. 51 (3) (2018) 1376–1388.
[41] R. Lomonaco, C. Ortiz-Lopez, B. Orsak, A. Webb, J. Hardies, C. Darland, J. Finch, A. Gastaldelli, S. Harrison, F. Tio, K. Cusi, Effect of adipose tissue insulin resistance on metabolic parameters and liver histology in obese patients with nonalcoholic fatty liver disease, Hepatology 55 (5) (2012) 1389–1397.
[42] P. Angulo, Nonalcoholic fatty liver disease, N. Engl. J. Med. 346 (16) (2002) 1221–1231.
[43] Y. Fu, S. Silverstein, J.N. McCutcheon, M. Dyba, R.G. Nath, M. Aggarwal, H. Coia, A. Bai, J. Pan, J. Jiang, B. Kallakury, H. Wang, Y.W. Zhang, G. Giaccone, A.R. He, F. L. Chung, An endogenous DNA adduct as a prognostic biomarker for hepatocarcinogenesis and its prevention by Theaphenon E in mice, Hepatology 67 (1) (2018) 159–170.
[44] B.P. Mihalas, G.N. De Iuliis, K.A. Redgrove, E.A. McLaughlin, B. Nixon, The lipid peroxidation product 4-hydroxynonenal contributes to oxidative stress-mediated deterioration of the ageing oocyte, Sci. Rep. 7 (1) (2017) 6247.
[45] V.N. Bochkov, O.V. Oskolkova, K.G. Birukov, A.L. Levonen, C.J. Binder, J. Stockl, Generation and biological activities of oxidized phospholipids, Antioxid. Redox Signal. 12 (8) (2010) 1009–1059.
[46] J. Adachi, S. Matsushita, N. Yoshioka, R. Funae, T. Fujita, S. Higuchi, Y. Ueno, Plasma phosphatidylcholine hydroperoxide as a new marker of oxidative stress in alcoholic patients, J. Lipid Res. 45 (5) (2004) 967–971.
[47] J. Adachi, N. Yoshioka, R. Funae, Y. Nagasaki, T. Naiot, Y. Uwno, Phosphatidylcholine hydroperoxide levels in human plasma are lower than previously reported, Lipids 39 (2004) 891–896.
[48] L.D. Renner, D.B. Weibel, Cardiolipin microdomains localize to negatively curved regions of Escherichia coli membranes, Proc. Natl. Acad. Sci. U. S. A. 108 (15) (2011) 6264–6269.
[49] S.M. Claypool, Y. Oktay, P. Boontheung, J.A. Loo, C.M. Koehler, Cardiolipin defines the interactome of the major ADP/ATP carrier protein of the mitochondrial inner membrane, J. Cell Biol. 182 (5) (2008) 937–950.
[50] K. Kashfi, R.L. Mynatt, E.A. Park, G.A. Cook, Membrane microenvironment regulation of carnitine palmitoyltranferases I and II, Biochem. Soc. Trans. 39 (3) (2011) 833–837.
[51] U. Schlattner, M. Tokarska-Schlattner, S. Ramirez, A. Bruckner, L. Kay, C. Polge, R. F. Epand, R.M. Lee, M.L. Lacombe, R.M. Epand, Mitochondrial kinases and their molecular interaction with cardiolipin, Biochim. Biophys. Acta 1788 (10) (2009) 2032–2047.
[52] K. Salic, E. Gart, F. Seidel, L. Verschuren, M. Caspers, W. van Duyvenvoorde, K. E. Wong, J. Keijer, I. Bobeldijk-Pastorova, P.Y. Wielinga, R. Kleemann, Combined treatment with L-carnitine and nicotinamide riboside improves hepatic metabolism and attenuates obesity and liver steatosis, Int. J. Mol. Sci. 20 (18) (2019).
[53] L.G. Boros, D.P. D'Agostino, H.E. Katz, J.P. Roth, E.J. Meuillet, G. Somlyai, Submolecular regulation of cell transformation by deuterium depleting water exchange reactions in the tricarboxylic acid substrate cycle, Med. Hypotheses 87 (2016) 69–74.
[54] L.G. Boros, T.Q. Collins, G. Somlyai, What to eat or what not to eat-that is still the question, Neuro Oncol. 19 (4) (2017) 595–596.
[55] A. Eguchi, E. Kostallari, A.E. Feldstein, V.H. Shah, Extracellular vesicles, the liquid biopsy of the future, J. Hepatol. 70 (6) (2019) 1292–1294.