



Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: <https://www.sciopen.com/journal/2097-0765>

Co-intake of lauric and linoleic acids exacerbates hepatotoxicity in the metabolic dysfunction-associated steatohepatitis

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ABSTRACT: Metabolic dysfunction-associated steatohepatitis (MASH), an advanced manifestation of metabolic dysfunction-associated steatotic liver disease (MASLD), affects approximately 30% of adults worldwide. Fatty acids, as important nutrients, are absorbed and utilized in a complex mixture daily. Medium-chain fatty acids are recommended for patients with lipid metabolism disorders due to their favorable metabolic properties and linoleic acid (LA-l, an essential fatty acid) is commonly consumed, but their combined effects in MASH remain unclear. Therefore, in this study, we employed multi-level models and lipidomics to investigate the combined effects of lauric acid (LA-m) and LA-l under normal and MASH conditions. The results revealed that co-intake of LA-m and LA-l exacerbated hepatotoxicity specifically in MASH, but not in healthy conditions, indicating metabolic status may determine susceptibility. Mechanistically, this combination increased membrane sphingolipid accumulation under the MASH diet, inducing endoplasmic reticulum stress, and ultimately triggering hepatotoxicity. Moreover, the combination increased ACSL4 expression to generate AA/AdA-PE, suggesting that LA-m may make hepatocytes more sensitive to ferroptosis by promoting the esterification of LA-l. Collectively, these findings provide caution in the concomitant use of LA-m and LA-l in MASH patients.

Keywords: Lauric acid; linoleic acid; metabolic dysfunction-associated steatohepatitis; endoplasmic reticulum stress; ferroptosis

1. Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) has been recognized as the number one chronic liver disease and a major global public health burden ^[1]. The global prevalence of MASLD is 30% and rising ^[2]. Metabolic dysfunction-associated steatohepatitis (MASH), an advanced manifestation of MASLD, is distinguished by the presence of hepatic steatosis, inflammation responses, hepatocellular injury, and fibrotic pathway activation. Moreover, the existence of MASH increases the risk of developing cirrhosis and hepatocellular carcinoma (HCC) ^[3-5]. Dietary and lifestyle-induced obesity, dyslipidemia, and type II diabetes have been proven to be risk factors for the development of MASH ^[6]. It is known that excessive free fatty acids, free cholesterol, diglycerides, ceramides, phospholipids, and other lipid

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Received 3 July 2025
Received in revised form 11 August 2025
Accepted 13 January 2026

metabolites in the liver can increase lipotoxicity, induce endoplasmic reticulum (ER) stress-mediated hepatic inflammatory response activation, mitochondrial dysfunction, and lipid peroxidative damage, resulting in the development of MASH, liver fibrosis, and HCC [7]. Finally, it has been reported that ceramides accumulate in the serum and liver of MASH patients [8]. Increasing evidence suggests that dietary interventions can affect the development of MASH.

Fat is one of the three major nutrients essential to the human body, holding a crucial role in numerous physiological processes, for example energy supply (9 kcal per gram), cell membrane components, and signaling molecules (e.g., activation of G-protein-coupled receptors (GPCR)) [9]. Fatty acids are the basic unit of fat composition. According to reports, different types of fatty acids have distinct roles in the progression of MASH [10].

Linoleic acid (LA-1, n-6) and α -linolenic acid (ALA, n-3), the two primary polyunsaturated fatty acid (PUFA), are essential fatty acids for humans and animals. Major source of n-6 are vegetable oils including safflower oil (75.7%) [11], soybean oil (51%), sunflower oil (65.7%), and corn oil (53.5%) [12], whereas n-3 PUFAs are mainly found in flaxseed oil and some fish [13]. It is estimated that the current Western diet is considered "lacking" in n-3 fatty acids, featuring an n-6 to n-3 ratio ranging from 15:1 to 16.7:1, rather than 1/1 ratio observed in the diets of wildlife and humans [14]. This imbalance has sparked debate about whether excessive consumption of linoleic acid is linked to the prevalence of chronic diseases. Some studies, such as those by Cortez-Pinto et al., suggested that elevated consumption of n-6 PUFA and an increased ratio of n-6/n-3 may contribute to the development of MASH [15]. Consistently, a lipidomic analysis revealed an upregulation ratio of the n-6/n-3 PUFAs in individuals diagnosed with MASH [16]. Of note, in a specific pathological context, linoleic acid has been proven to exacerbate chronic disease progression. For instance, excessive intake of linoleic acid has been shown to worsen ethanol-induced liver injury [17], aggravate alcohol-induced enteritis [18], and intensify high-fat diet (HFD) and streptozotocin-induced metabolic responses and gut microbiota dysbiosis [19]. On the contrary, some studies have shown that increased intake of linoleic acid in healthy populations has a significant negative correlation with the occurrence of chronic diseases, including type 2 diabetes [20], cardiovascular diseases [21] and MASLD [22, 23], etc. These findings suggest that the role of linoleic acid may be context-dependent and influenced by individual metabolic status.

Lauric acid (LA-m) is a medium-chain saturated fatty acid, primarily found in coconut oil (45%-53%) [24] and milk fat (2.5%-4%) [25]. As an important dietary component, medium-chain fatty acids (MCFAs) can be directly transported to the liver via the portal bloodstream to rapidly metabolize without forming chylomicrons in the enterocytes. The unique metabolic properties of MCFAs can provide the body with an alternative source of energy or fatty acids. Therefore, the diseases related to abnormal fat metabolism, such as long-chain fatty acid oxidation disorder, lipodystrophy, and impaired lymphatic chylomicron transport, could be controlled or alleviated by replacing most long-chain fatty acids in diet with MCFAs [26]. In metabolic disorders such as diabetes, obesity, and hypertriglyceridemia, MCFAs improve metabolic homeostasis mainly by promoting lipid metabolism and weight loss [27]. In MASH disease, endogenous MCFAs (C10:0 or C12:0,

but not C8:0) were effective in improving MASH mediated by GPR84 in a mouse model [28]. However, in the MASLD cohort, higher plasma lauric acid was significantly correlated with higher MASH activity scores [29].

In summary, the role of linoleic acid in regulating MASH may depend on the metabolic status of the patients. MCFAs and linoleic acid have been recommended to be used for patients with lipid metabolic disorders considering that MCFAs have favorable metabolic features while linoleic acid is an essential fatty acid [26, 30]. Therefore, it is necessary to evaluate the effects of co-intake of lauric and linoleic acids in healthy and MASH mice, respectively. Interestingly, we found that the combination of lauric acid (a representative of MCFAs) and linoleic acid specifically exacerbated the progression of liver injury, in both models of MCD medium and MASH mice. However, this had no effect on healthy mice. The findings of this study provide potential risk warning concerning fatty acid consumption for individuals with metabolic disorders especially MASH.

2. Materials and Methods

2.1. Ethics statement

All experiments involving animals were conducted according to the ethical policies and procedures approved by the ethics committee of China Agricultural University (Approval no. Aw42604202-4-6).

2.2. Chemicals and antibodies

Lauric acid (LA-m) (purity $\geq 99.98\%$), linoleic acid (LA-l) (purity $\geq 99.92\%$), 4 μ 8C (IRE1 α inhibitor), SP600125 (JNK inhibitor), and liproxstatin-1 (Lip-1, ferroptosis inhibitor) were obtained from MedChemExpress (Monmouth Junction, NJ, USA). Sphingolipid inhibitor myriocin (ISP-1) was acquired from Aladdin (Shanghai, China). Liperfluo (LPO) was purchased from DOJINDO (Kyushu, Japan). Phospho-PERK (82534-1-RR) was purchased from Proteintech (Wuhan, China). PERK (#3192), phospho-eIF2 α (#3398), eIF2 α (#5324), IRE1 α (#3294), phospho-JNK (#4668) and JNK (#9252) antibodies were from Cell Signaling Technology (Beverly, MA, USA). Phospho-IRE1 α (#ab48187) and TFR1 (#ab214039) were purchased from Abcam (Cambridge, MA, USA). ACSL4 (sc-365230) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody targeting β -actin and the Rabbit and Mouse specific secondary antibodies were both from MBL International Corporation (Beijing, China).

2.3. Cell culture and treatments

AML12 and HepG2 cell lines were provided by the American Type Culture Collection (ATCC). AML12 and HepG2 cells were respectively cultured in DMEM/F12 plus 1% ITS and DMEM medium (Hyclone, Logan, UT, USA) contained with 10% fetal bovine serum without antibiotics. All cultures were maintained with 5% CO₂ at 37 °C. Cells were cultivated for 24 h until they reached a confluence of 40% to 50%. Subsequently, cells were incubated in methionine-choline-sufficient (MCS, PM150310, Pricella) and methionine-choline-deficient (MCD, DM150310, Pricella) media with different agents.

2.4. Isolation of primary hepatocytes

Primary hepatocytes were isolated from 6-week-old C57BL/6J Nifdc mice through a two-step collagenase perfusion method, as previously described [31]. The isolated hepatocytes were cultivated in William's Medium E supplemented with 10% fetal bovine serum and 1% (v/v) penicillin/streptomycin, maintained at 37°C with 5% CO₂, and plated on plates coated with rat tail tendon collagen type I. Then, cells were exposed to LA-m or LA-l or the combination for the indicated time in MCS and MCD media.

2.5. Construction of liver organoids

Primary human hepatocytes (HUCPI-HUM181651) were purchased from Lonza Verviers SPRL (Verviers, Belgium), and informed consent was provided from all patients. All reagents for hepatocyte culture were purchased from Beijing Daxiang Biotech Co., Ltd. After thawing, the hepatocytes were mixed with Matrigel, and seeded in a 24-well/plate. After Matrigel had solidified, an organoid expansion medium (EM) containing growth factors was added and incubated at 37°C, 5% CO₂. The medium was changed every three days. After 5-6 days, the EM was removed, and the liver organoids were differentiated for 5 days in a differentiation medium (DM). When the liver organoids were fully differentiated, the organoids were then treated with LA-m or LA-l or the combination for 24 h in MCS and MCD media, respectively.

2.6. Cell viability assessment

Cell viability was assessed using crystal violet staining, Cell Counting Kit-8 (CCK-8), and CellCounting-Lite 3D, all in accordance with the manufacturer's guidelines.

2.7. Lipid peroxides measurement

After treatment, cells were collected and then incubated with 1 µM Liperfluo for 30 minutes at 37 °C. After incubation, the fluorescence intensity of the cells was detected by flow cytometry.

2.8. Immunofluorescence staining

Liver organoids (diameter > 50 µm) were harvested and then fixed, washed, blocked, incubated with antibodies and photographed by fluorescence. The primary antibodies for HNF4α (#3113) and MRP2 (#4446) were from Cell Signaling Technology (Beverly, MA, USA). The Rabbit specific secondary antibody was obtained from Servicebio company. DAPI and F-actin were used to stain the nucleus and cytoskeleton of cells.

2.9. Animal experiments

The seven-week-old male C57BL/6J Nifdc mice were acquired from Charles River Laboratory (Beijing, China). A total of 90 mice were allowed one week of acclimatization with ad libitum water and food in specific pathogen-free conditions (temperature: 22 ± 2 °C; humidity: 55 ± 10%; 12-h day/night cycle). Then, the mice were randomly assigned to the normal diet group (ND, n = 40), methionine-choline-sufficient diet group (MCS, n = 10), and methionine-choline-deficient diet group (MCD, n = 40). The experimental scheme is as follows:

ND group: The mice were divided at random into four groups (n = 10/per group). Three groups of mice were intragastrically administered with 350 mg/kg bw/day LA-m or 1000 mg/kg bw/day LA-l or combination for 3 weeks. The control group was gavaged with solvent at the same body weight ratio.

MCD group: The experimental group and administration period were the same as those of the ND group, and the MCS group was used as the control.

The body weight was weighed every other day, and food intake was also monitored. After 3 weeks, all mice were euthanized, and blood and liver samples were immediately collected.

2.10. Biochemical analysis

The hepatic non-esterified fatty acid (NEFA) and triglyceride (TG), serum alanine transaminase (ALT) and aspartate aminotransferase (AST) levels were measured according to the manufacturer's guidelines from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.11. Histopathology

Liver tissue was fixed, embedded, and sectioned for haematoxylin and eosin (H&E) and Oil Red O staining. For MASH activity score was estimated as described previously^[32]. The Image-pro plus 6.0 software was utilized to quantify the areas occupied by hepatic lipid droplets.

2.12. Western blotting

The cells and mouse liver tissues underwent lysis in ice-cold RIPA buffer, which contained both protease and phosphatase inhibitors. Subsequently, equivalent quantities of protein samples were separated by SDS-PAGE and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA; IPVH00010). Following blocking with 5% non-fat dry milk, the membranes were incubated overnight at 4 °C with primary antibodies. Afterward, they were incubated for 1 h at room temperature with an HRP-conjugated secondary antibody. To detect protein expression, enhanced chemiluminescence was utilized.

2.13. Lipidomics Analysis

The MTBE method was employed for extracting lipids from the liver. Briefly, water, pre-cooled methanol, and MTBE were sequentially added to the samples. Then, the solution was centrifuged and separated the upper organic solvent layer, and dried using nitrogen. The lipid extracts were then re-dissolved and separated by reverse phase chromatography with an injection volume of 3 µL. Acetonitrile-isopropanol (1:9, v/v) was used as the initial mobile phase and the flow rate was 300 µL/min for 35 min. Subsequently, mass spectrometry data were obtained using the Q-Exactive Plus instrument, in both positive and negative ion modes.

2.14. Statistical Analysis

GraphPad Prism 8.0 was used for statistical analysis. All results were expressed as means ± SD and subjected to either one-way or two-way ANOVA, with subsequent appropriate post hoc tests to compare the means. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Hepatocytes cultured in MCD medium are susceptible to co-exposure of LA-m and LA-l- induced toxicity

To investigate the effects of metabolic status on the sensitivity of liver cells to LA-m/LA-l-induced lipotoxicity, AML12, HepG2, and mouse primary hepatocytes cultured in either MCS or MCD medium were employed to investigate the changes of cell viability in response to LA-m and LA-l alone or their combination. These hepatocytes underwent treatment with different concentrations of LA-m and/or LA-l for 24 h, and cell viability was measured by crystal violet staining and CCK-8 assay. As illustrated in Figure 1A and B, under MCS culture conditions, AML12 cells exhibited no significant change in viability when treated with each fatty acid alone or the combination compared to the control group. Under MCD culture conditions, individual fatty acids did not result in significant alterations in cell viability; however, a significant reduction of cell viability was induced by the co-treatment of LA-m and LA-l. Similar results, particularly, the enhanced reduction of cell viability by combination of LA-m and LA-l were also found in HepG2 cells (Figure 1C, and D) and primary mouse hepatocytes (Figure 1E-G). Taken together, these results suggest that MCD medium-induced metabolic disorder rendered liver cells susceptible to the co-exposure of LA-m and LA-l induced toxicity.

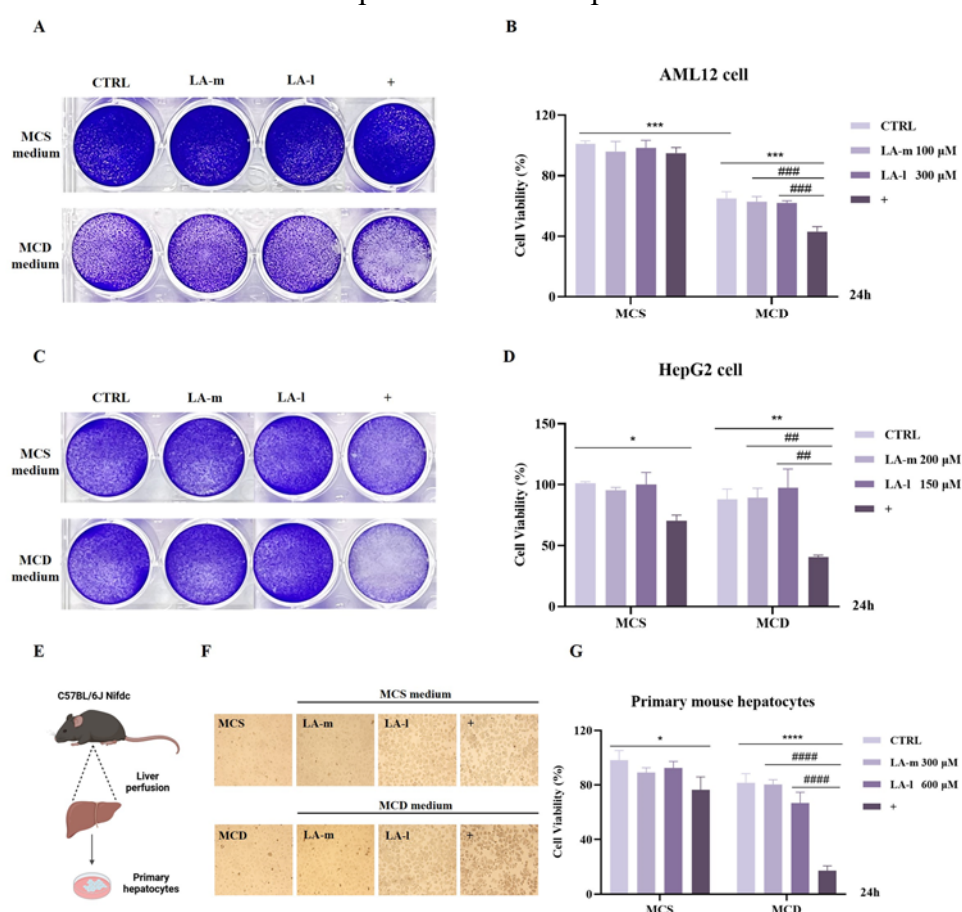
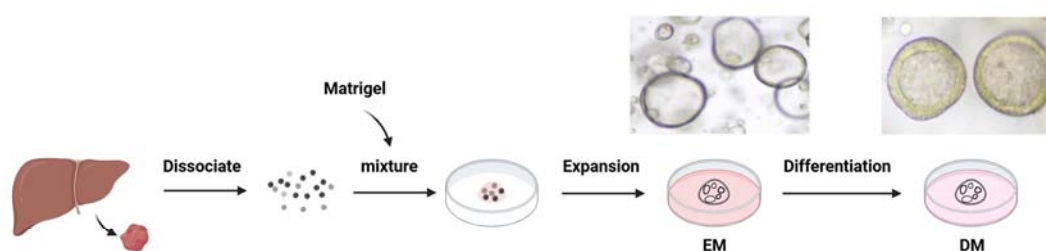


Fig.1 Hepatocytes cultured in MCD medium are sensitive to the toxicity induced by the combination of LA-m and LA-l. (A, B) AML12 cells and (C, D) HepG2 cells were exposed to LA-m and LA-l alone or their combination in MCS and MCD media for 24 h, and cell viability changes were assessed through crystal violet staining. (E) Schematic illustration of primary hepatocytes isolation from C57BL/6J Nifdc mice. (F) and (G) Mouse primary hepatocytes were subjected to specified concentrations of LA-m and LA-l alone or their combination in MCS and MCD media for 24 h, and then their viability was assayed using the CCK8. Results are displayed as the mean $\pm$ SD (n = 3), * P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001 versus the MCS or MCD group. ### P < 0.01, #### P < 0.001 and ##### P < 0.0001 versus the LA-m or LA-l group.

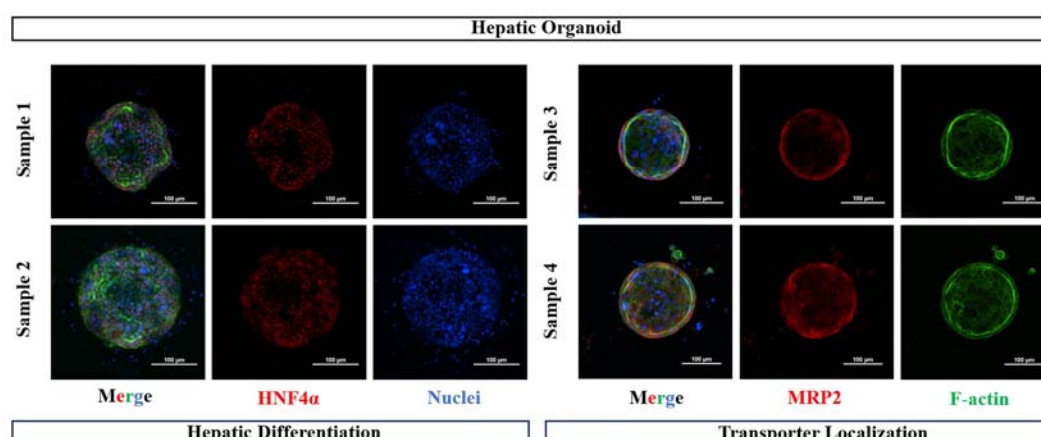
3.2. Human hepatic organoids cultured in MCD medium are susceptible to combined of LA-m and LA-l-induced toxicity

To more realistically simulate the human environment, we performed the similar experimental operation on human-derived hepatic organoids. The hepatic organoids are 3D cell clusters based on the redifferentiation and self-assembly of human primary hepatocytes. It is a highly biomimetic model of the liver *in vitro* with high genetic and histological homogeneity with liver tissue, which can be used for long-term or acute drug hepatotoxicity assays and related studies. Firstly, hepatic organoids need to undergo expansion and differentiation stages before performing experiments (Figure 2A). The immunofluorescence results showed that the characteristic transcription factor HNF4 α (red marker) of hepatocytes in hepatic organoids was highly expressed in the nucleus (blue marker), indicating that the hepatic organoids expanded well. Meanwhile, MRP2 (red marker), a transporter characteristic of mature hepatocytes, was also highly expressed in the differentiated cultured hepatic organoids and correctly polarized at the outer edge of the organoids, suggesting that human-derived hepatic organoids at the expansion stage further differentiated into more mature hepatic organoids in the differentiation medium (Figure 2B). Next, mature hepatic organoids were simultaneously exposed to LA-m and LA-l in MCS and MCD media, respectively, and the data are presented in Figure 2C and D. In agreement with the result found in cultured liver cells, no changes in cell viability in organoids in response to each fatty acid alone or the combination were observed under MCS culture condition, whereas the combination of LA-m and LA-l led to a notable reduction in cell viability in hepatic organoids under MCD culture condition. These results provided more clinically relevant evidence for the hepatotoxicity induced by co-exposure of LA-m and LA-l under condition of MCD medium-mediated metabolic disorder.

A



B



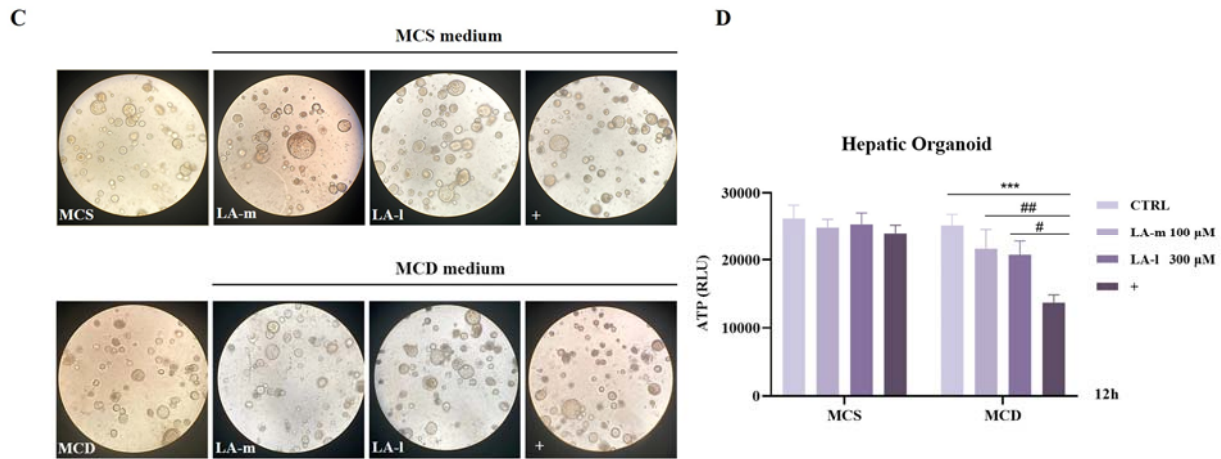
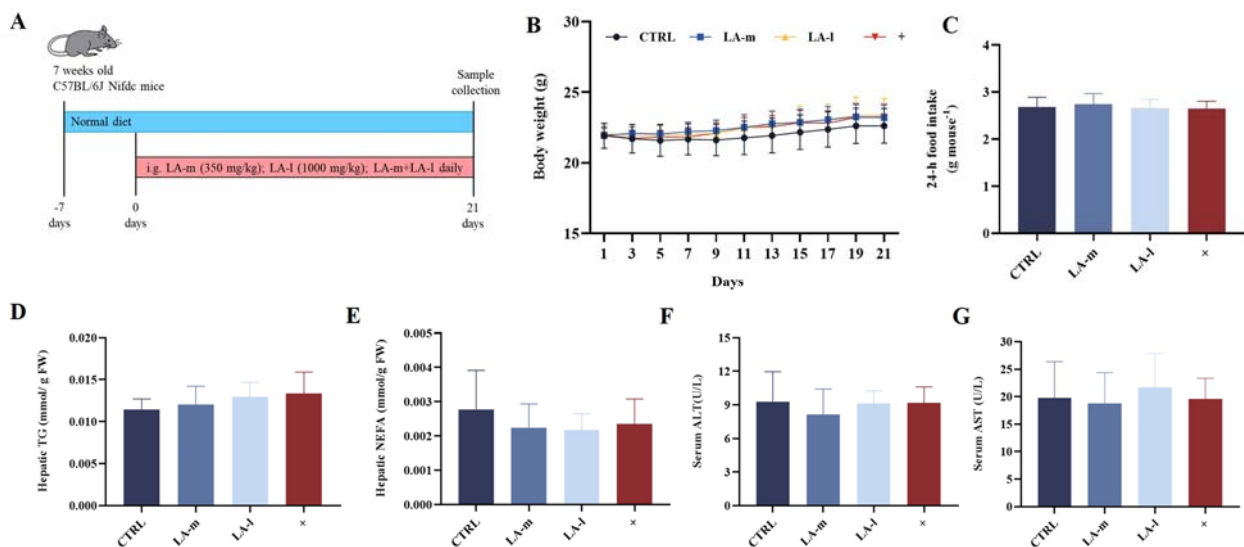


Fig.2 Human hepatic organoids cultured in MCD medium are susceptible to toxicity induced by co-exposure of LA-m and LA-l. (A) Diagram of constructing liver organoids from primary human hepatocytes. EM: expansion medium; DM: differentiation medium. (B) Hepatic differentiation (HNF4 α) and transporter localization (MRP2) were evaluated by immunofluorescence staining. Four liver organoids in the field of view were randomly selected for photographing. DAPI: nuclei; F-actin: cytoskeleton. (C, D) Hepatic organoids cultured in the MCS and MCD media with treatment of LA-m and LA-l alone or their combination for 12 h, and cell viability was assessed by CellCounting-Lite 3D. Results are expressed as mean $\pm$ SD (n = 3), *** P < 0.001 versus the MCD group. # P < 0.05 and ## P < 0.01 versus the LA-m or LA-l group.

3.3. LA-m and LA-l combination worsens MCD diet-induced MASH, but has no effect in mice fed with ND

Having found the hepatotoxicity of LA-m and LA-l co-exposure in the MCD medium cultured hepatocytes and hepatic organoids, we wondered whether this toxic effect could manifest *in vivo*. We first examined whether combination of LA-m and LA-l caused hepatotoxicity in healthy mice. A concise illustration of the experimental design is presented in Figure 3A. Seven-week-old male C57BL/6J Nifdc mice fed with normal diet were treated with LA-m (350 mg/kg) and LA-l (1000 mg/kg) alone or the combination for 3 weeks (the doses used in this study reflect realistic exposure levels for certain populations), and the changes in body weight, food intake, hepatic TG, hepatic NEFA, and serum ALT/AST were analyzed. As shown in Figure 3B-G, neither LA-m and LA-l alone nor the combination induced abnormal changes of these parameters. These results suggest that LA-m and LA-l alone or their combination are not hepatotoxic to healthy mice, which was further supported by H&E and Oil red O staining of liver tissues (Figure 3H and I).



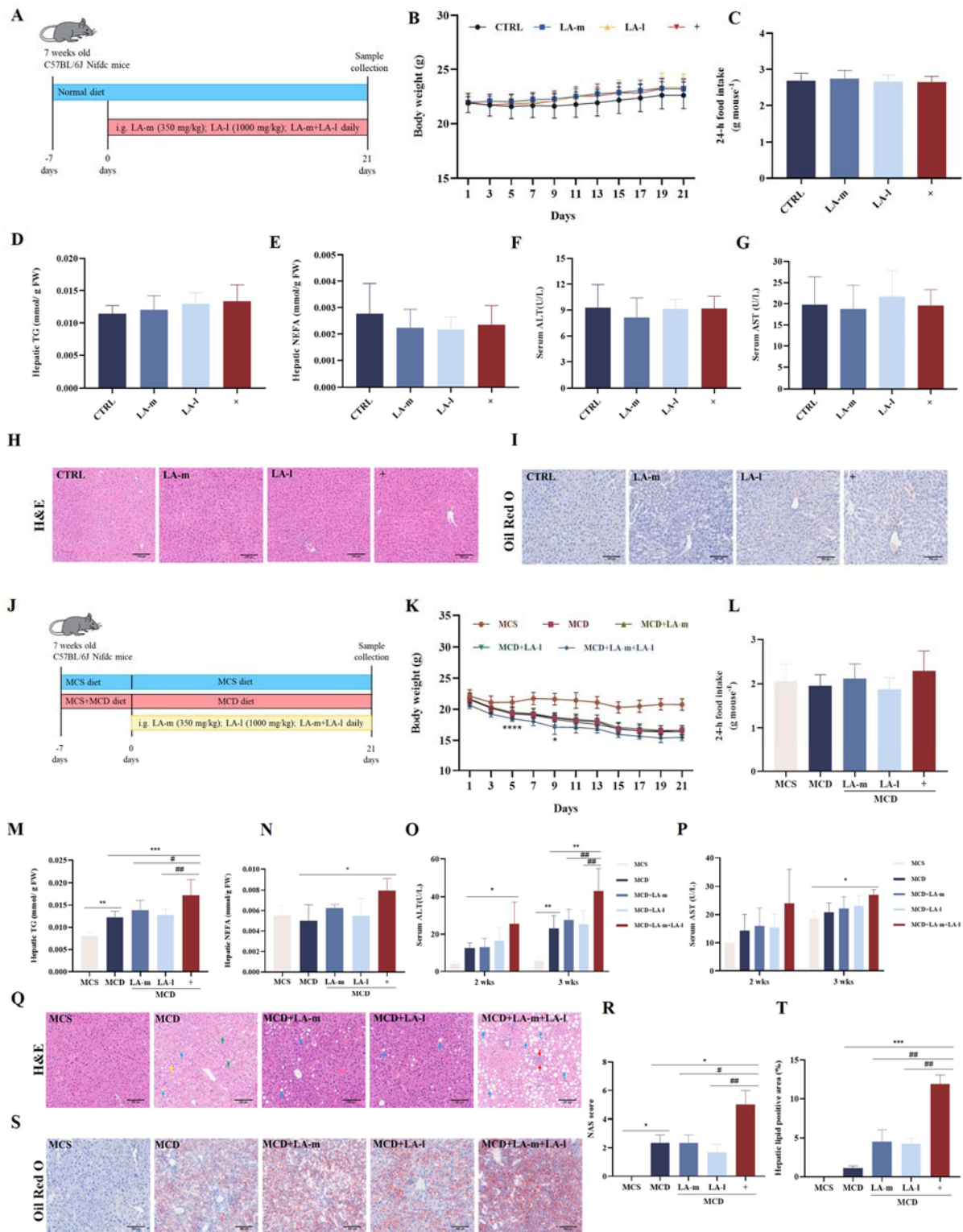


Fig.3 LA-m and LA-l combination aggravates hepatic steatosis and hepatotoxicity induced by an MCD diet, but has no effect in mice fed with ND. ND group: (A) Illustrative representation of the experimental design for mice fed with a ND diet. (B) Body weight measured every other day during the study period. (C) Average food intake. (D-G) End of experiment levels of (D) hepatic triglyceride (TG), (E) hepatic non-esterified fatty acids (NEFA), (F) serum alanine aminotransferase (ALT) and (G) serum aspartate aminotransferase (AST). (H-I) Histological staining of liver sections with H&E (H) and Oil Red O (I); magnification $\times 200$. Scale bars: 100 μm . MCD diet group: (J) Schematic diagram of the experimental design in MCD-fed mice. (K) Body weight measured every other day for 3 weeks. (L) Average food intake. (M-P) End of experiment levels of (M) hepatic triglyceride (TG), (N) hepatic non-esterified fatty acids (NEFA), (O) serum alanine aminotransferase (ALT) and (P) serum aspartate aminotransferase (AST). (Q-T) H&E staining (Q) and liver NAS scores (R), estimated on liver sections; Oil Red O staining (S) and hepatic lipid positive area (T) of liver sections; magnification $\times 200$. Scale bars: 100 μm . Results are shown as mean $\pm$ SD ($n = 6/\text{group}$), $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ versus the MCS or MCD group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the LA-m or LA-l group.

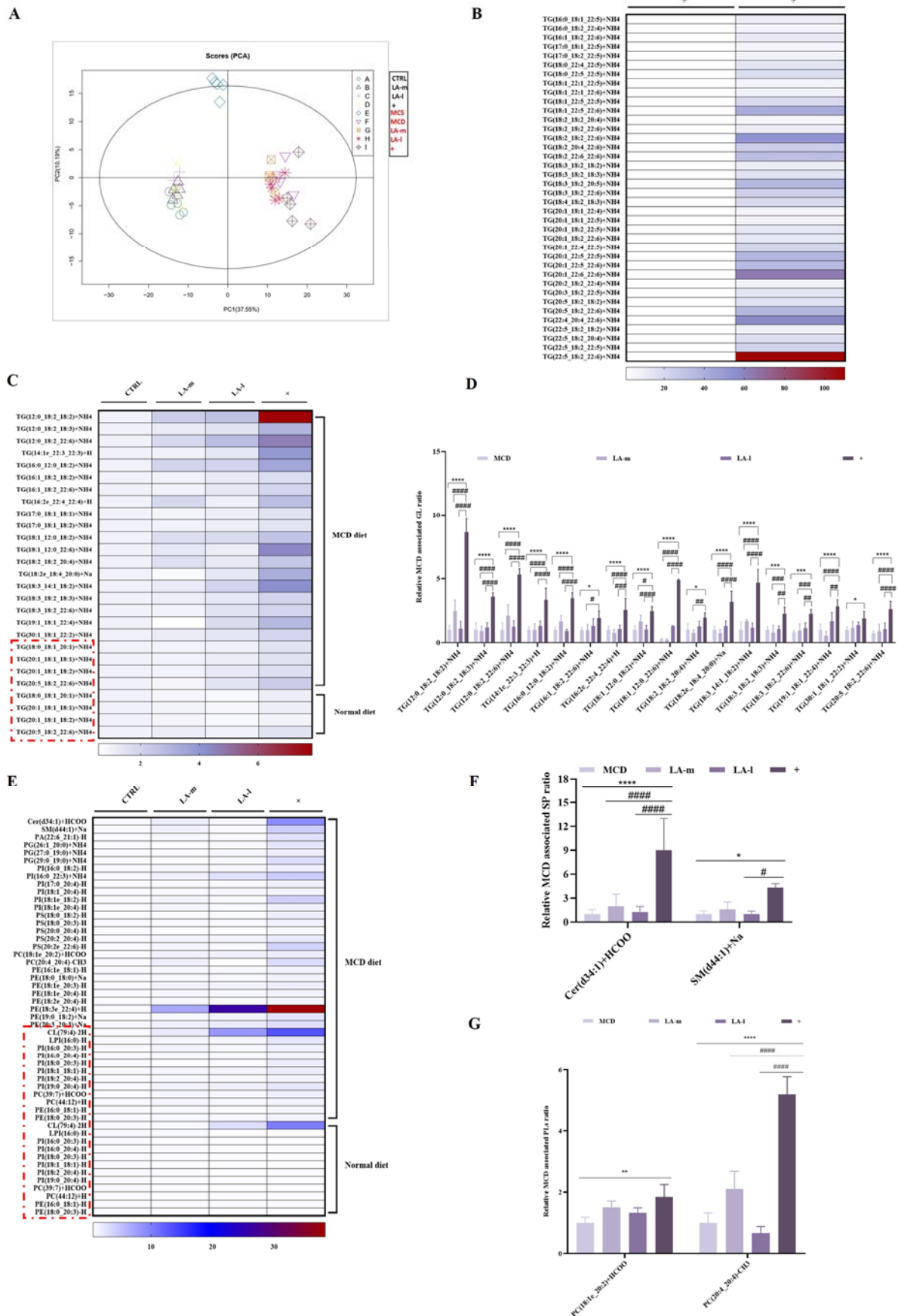
We next assessed if combination of LA-m and LA-l caused hepatotoxicity in MCD diet-induced MASH mice. The experimental design is shown in Figure 3J. Seven-week-old male C57BL/6J Nifdc mice fed with MCS or MCD diet were treated with LA-m (350 mg/kg) and LA-l (1000 mg/kg) alone or the combination for 3 weeks, and the alternations in body weight, food intake, lipid metabolisms and liver injury markers were evaluated. Mice fed with the MCD diet exhibited a significantly lower body weight compared to those in the MCS group ($P < 0.0001$), and combined exposure to LA-m and LA-l induced further reduction of body weight in MCD diet-fed mice ($P < 0.05$), as illustrated in Figure 3K. There were no significant differences in food intake among these groups (Figure 3L). The MCD animal model is caused by dietary deficiencies of choline and methionine, which further triggers impaired synthesis of very low-density lipoproteins (VLDL) in the body, ultimately leading to massive fat accumulation in the liver^[33]. As expected, mice fed with the MCD diet had an increased content of hepatic TG compared to those fed with the MCS diet, whereas a further elevated hepatic TG content was induced by combination of LA-m and LA-l compared with MCD diet control or each fatty acid treatment alone (Figure 3M). Moreover, combination of LA-m and LA-l but not each alone led to a significant elevation in hepatic NEFA levels ($P < 0.05$, Figure 3N) and serum ALT/AST levels (Figure 3O and P), the two liver injury markers, in mice fed with MCD diet. Further, we performed histological analyses of the liver tissue and results are shown in Figure Q. H&E staining showed that MCD-diet feeding led mild steatosis (blue arrows), some of the hepatocytes were edematous, with pale staining of the cytoplasm (yellow arrows), and a small number of the cytoplasm is vacuolated (green arrows) compared to the MCS group. LA-m or LA-l administration alone triggered moderate steatosis (blue arrows), while the combination induced more severe hepatic steatosis (blue arrows), and a few inflammatory cell infiltrations (red arrows), as compared with the MCD group. This result is well consistent with NAS scores (Figure 3R). Meanwhile, Oil red O staining revealed that all MCD treatments caused hepatic lipid deposition (vs. MCS), which appeared to be greater with LA-m and LA-l co-exposure (Figure 3S, and T).

Taken together, the combination of LA-m and LA-l dramatically worsened MCD diet-induced hepatic steatosis and hepatotoxicity, but had no effect on these parameters in ND-fed mice.

3.4. Hepatic lipid metabolism disruption is involved in LA-m and LA-l co-exposure-exacerbated hepatotoxicity

To decipher the mechanisms involved in combination of LA-m and LA-l -induced hepatotoxicity, LC-MS/MS-based redox lipidomics was conducted to examine alterations in lipid metabolism induced by each fatty acid alone or their combination under either healthy or MCD diet-induced MASH condition. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were used to elucidate the differences and separation in the hepatic lipid metabolism profiles among various treatment groups. PCA showed distinct lipid clusters between ND-fed and MCD diet-fed groups (Figure 4A). OPLS-DA revealed a clear separation of lipid profiles of mice fed ND or MCD diet, and confirmed model stability by judging Q^2 values (Figure S2A, and B). As expected, relative quantification results demonstrated a notable elevation in hepatic TG levels in response to the MCD diet compared to the MCS diet, thereby confirming the effectiveness of the MCD diet in inducing the MASH model (Figure 4B). Under the MCD diet,

the combination not only caused an increase in TG species but also led to more severe elevations for the same type of TG (red dotted box), in contrast to ND (Figure 4C, and D). This result is consistent with the TG results measured by biochemical kits.



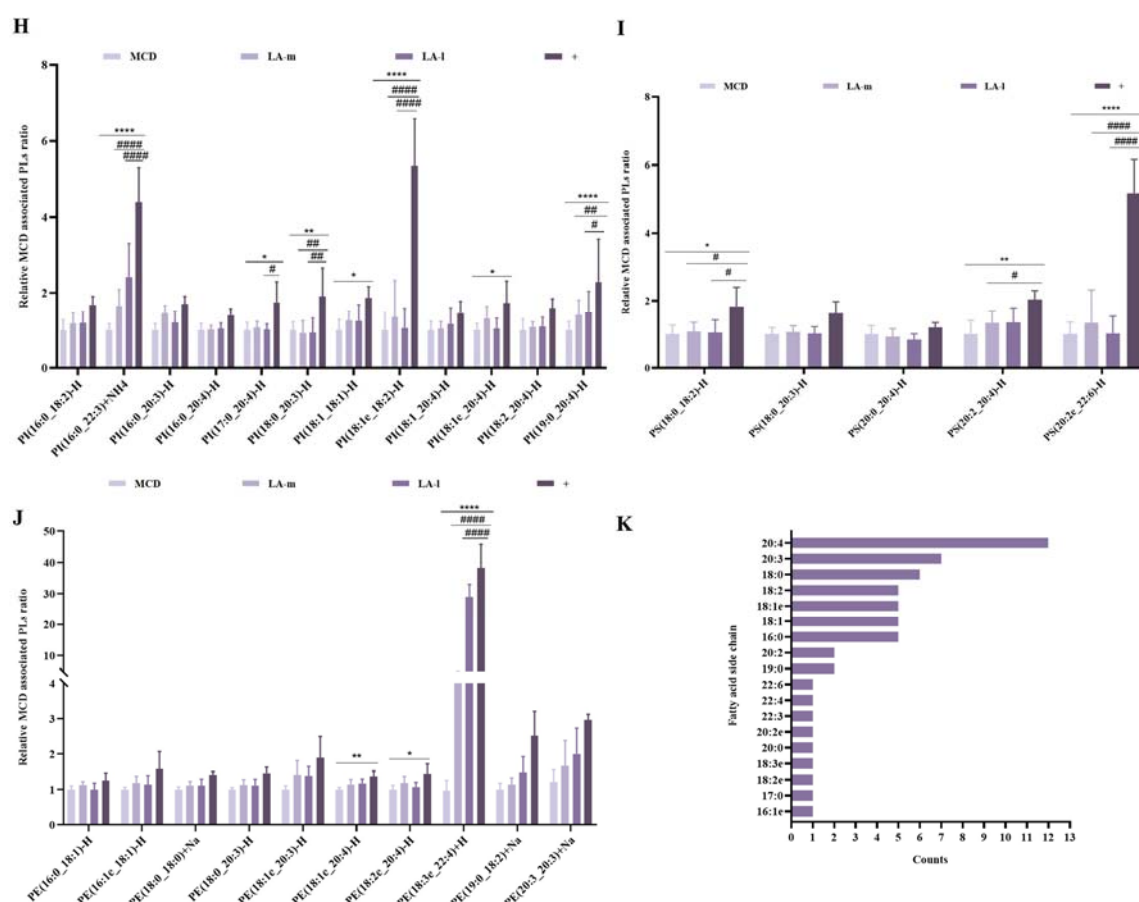


Fig.4 LA-m and LA-l combination induces triglyceride, sphingolipids, and glycerophospholipids accumulation in MCD-fed mice, thereby exacerbating hepatotoxicity. (A-K) Effect of LA-m and LA-l combination on liver phospholipids.

Mice treated with LA-m (350 mg/kg) and LA-l (1000 mg/kg) alone or their combination for 3 weeks. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to analyze lipid composition. (A) Principal component analysis (PCA) of lipid distribution in ND and MCD diet-fed mice. (B) Heatmap shows triglyceride (TG) fold-changes in mice fed MCD diet compared to MCS diet. (C) Heatmap shows TG fold-changes in LA-m and LA-l alone or their combination under MCD and ND condition, respectively. (D) The relative changes of different glycerolipid (GL) (C and D) are shown in MCD diet-fed mice ($n = 5$). (E) Heatmap shows sphingolipid (SP) and glycerophospholipid (GP) fold-changes in LA-m and LA-l alone or their combination under MCD and ND condition, respectively. (F-J) The relative changes of different SP and GP (E), Cer and SM (F), PC (G), PI (H), PS (I), PE (J) are shown in mice fed MCD diet ($n = 5$). (K) The statistics of the fatty acid side chain of lipids. Results are indicated as mean $\pm$ SD ($n = 5$ /group), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ versus the MCD group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ and #### $P < 0.0001$ versus the LA-m or LA-l group. Ceramide (Cer), sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidic acid (PA).

The effect of LA-m and LA-l co-exposure on the alterations in lipid species was further explored, and differential lipid molecules with an OPLS-DA VIP score exceeding 1 and a P value less than 0.05 were screened. Treatment with LA-m plus LA-l changed the relative abundance of different lipid species in MCD-fed mice compared to ND (Figure 4E). Next, the sphingolipid (SP) composition was analyzed under MCD diet feeding. A marked rise in levels in the liver ceramide (Cer, d34:1) and sphingomyelin (SM, d44:1) of the combination group mice compared with MCD alone (Figure 4F). Glycerophospholipids (GPs) are the most abundant phospholipid components in the body, which are one of the main components of cell membranes and play a key role in fat metabolism. A lipidomic analysis uncovered that the levels of GPs (including PC, PI, PS, PE, etc.) were generally increased in the combination-treated mice fed the MCD diet (Figure 4G-J). Furthermore, fatty acid side chain containing adrenic acid (AdA) (22:4) was preferentially

integrated into PE (Figure 4J). Further analysis indicates that arachidonic acid (AA, 20:4) predominates in their fatty acid side chains (Figure 4K).

Collectively, supplementation with LA-m plus LA-l altered lipid metabolism, resulting in under MCD diet-induced MASH condition, which might contribute to the combination-induced hepatotoxicity.

3.5. Sphingolipids accumulation-caused endoplasmic reticulum stress is a primary driver of hepatotoxicity by LA-m and LA-l combination in vitro and in vivo

Redox lipidomics analysis demonstrated that LA-m and LA-l combination resulted in accumulation of sphingolipids in liver in MCD diet-induced MASH model. To determine the contribution of sphingolipid accumulation to combination of LA-m and LA-l-induced hepatotoxicity, we assessed influences of inhibition of de novo synthesis of sphingolipids by ISP-1, an inhibitor of SPT (a key enzyme in synthesis of sphingolipids) on the HepG2 cells toxicity induced by combination of LA-m plus LA-l. As illustrated in Figure 5A, the reduction of cell viability induced by combination of LA-m and LA-l was significantly attenuated in the presence of ISP-1, supporting the contribution of SP accumulation to the toxicity of LA-m/LA-l under MASH condition.

Studies have demonstrated that the induction of ER stress was involved in lipotoxicity. Additionally, Zhang et al.'s research indicates that endoplasmic reticulum stress plays a crucial role in arachidonic acid-induced ferroptosis in hepatocytes^[34]. Thus, we hypothesize that the hepatotoxicity by the combination of LA-m plus LA-l might be attributed to the enhancement in ER stress induction. We then analyzed the changes of ER stress markers and results are presented in Figure 5B and C. The relative expression of p-eIF2 α /eIF2 α , p-IRE1 α /IRE1 α , and p-JNK/JNK, three key ER stress markers, was significantly induced by the combination. Furthermore, inhibition of IRE1 α or JNK by its inhibitor rescued the cell viability reduction in both AML12 and HepG2 cells (Figure 5D and E).

To determine the relationship between sphingolipid accumulation and ER stress activation, we measured the impact of ISP-1 on key indicators of ER stress. As shown in Figure 5F, p-IRE1 α /IRE1 α and p-JNK/JNK were repressed in the presence of ISP-1, suggesting ER stress activation lying downstream of SP accumulation. Moreover, the enhanced ER stress activation by the combination was detected in liver tissues of MCD diet-induced MASH mice (Figure 5G, and H). However, it was absent in mice induced with ND (Figure 5I). These results indicate that sphingolipid accumulation-mediated ER stress activation play a pivotal role in combination of LA-m and LA-l-induced hepatotoxicity.

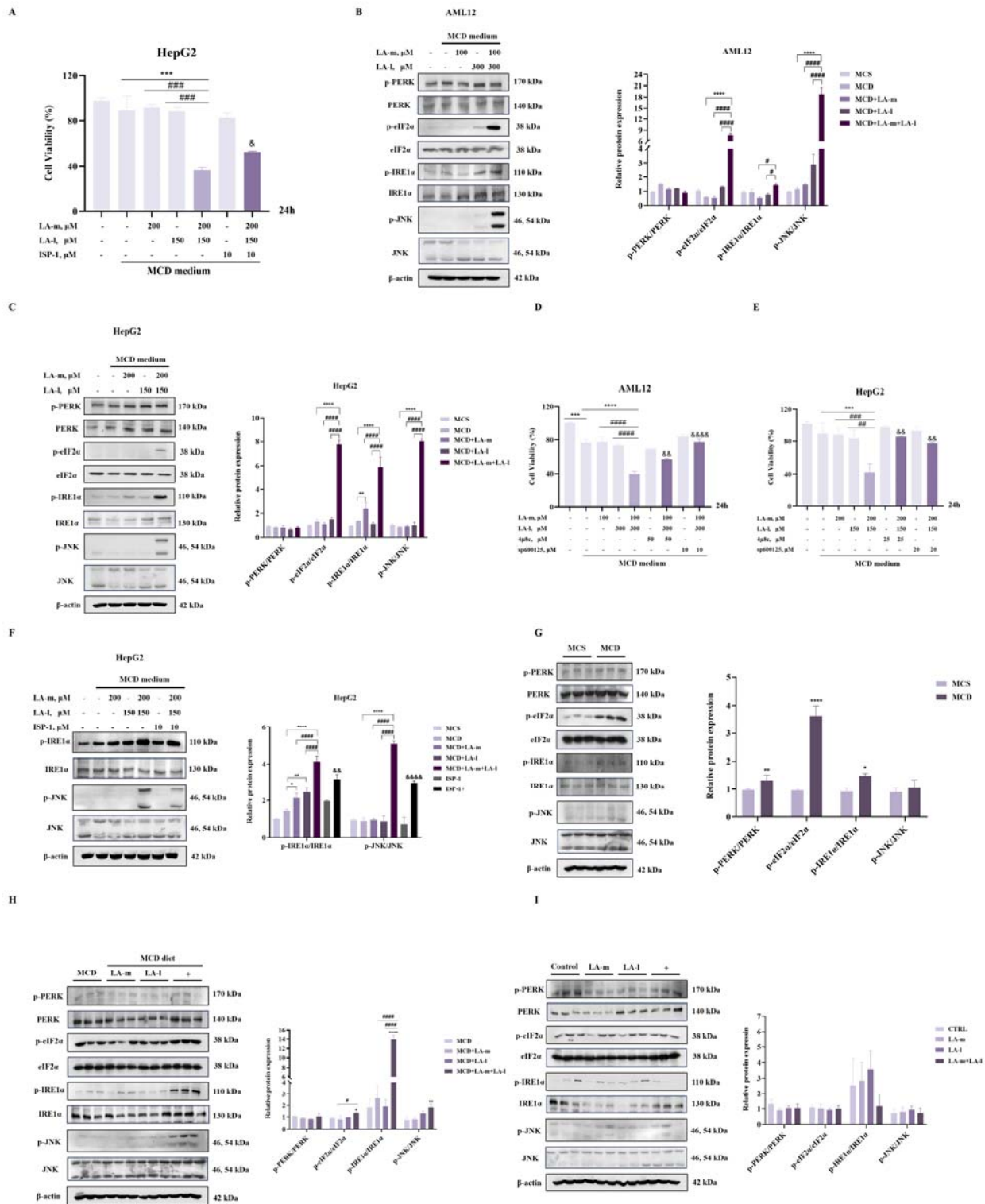


Fig.5 The combination of LA-m plus LA-l induces sphingolipids accumulation under MCD condition, further upregulating the levels of ER stress-related proteins *in vitro* and *in vivo*. (A) HepG2 cells were treated with LA-m and LA-l alone or their combination in the absence or presence of ISP-1 for 24 h, and then cell viability was determined. (B and C) The relative expression and quantitative analysis of p-PERK/PERK, p-eIF2 α /eIF2 α , p-IRE1 α /IRE1 α , and p-JNK/JNK were detected by western blotting for 24 h after LA-m and LA-l alone or their combination treatment in AML12 (B) and HepG2 cells (C). (D and E) AML12 (D) and HepG2 cells (E) were treated with LA-m and LA-l alone or their combination in the absence or presence of 4 μ 8c and sp600125 for 24 h, and cell viability was determined. (F) The levels of p-IRE1 α /IRE1 α and p-JNK/JNK in HepG2 cells with or without treatment with ISP-1 or LA-m and LA-l alone or their combination for 24 h. (G-I) p-PERK/PERK, p-eIF2 α /eIF2 α , p-IRE1 α /IRE1 α , and p-JNK/JNK protein levels in MCD diet (G and H) and ND-fed (I) mice liver tissue lysates, as measured by western blotting. Results are shown as mean $\pm$ SD (n = 3), * P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001 versus the MCS or MCD group. # P < 0.05, ## P < 0.01, ### P < 0.001, and #### P < 0.0001 versus the LA-m or LA-l group. & P < 0.05, && P < 0.01, and &&& P < 0.0001 versus the LA-m + LA-l group.

3.6. Ferroptosis contributes to the co-exposure to LA-m and LA-l-exacerbated MASH progression

Ferroptosis represents a type of cell death that is fueled by the peroxidation of lipids. Phospholipids containing AA/AdA-PE are key membrane phospholipids that can be oxidized to toxic phospholipid hydroperoxides (PE-AA/AdAOOH) via enzymatic reactions, leading to ferroptosis [35]. Our lipidomics results showed that the combination of LA-m and LA-l led to a significant increase in AA/AdA-PE content under the MCD diet, implying that the combination-induced hepatotoxicity may be related to ferroptosis induction. Therefore, the ferroptosis-related proteins ACSL4 and TFR1 in AML12 and HepG2 cells were examined. As shown in Figure 6A and B, in the presence of the combination, the ACSL4 and TFR1 levels were significantly elevated compared with MCD medium alone. Similar results were found in mice fed with MCD diet (Figure 6C, and D). However, this combined effect was absent in ND-fed mice (Figure 6E). Furthermore, inhibition of ferroptosis by Lip-1 partially restored the cell viability decrease in AML12 cells (Figure 6F), which was further supported by measuring the levels of lipid peroxides (LPO) in AML12 cell and HepG2 cells, respectively (Figure 6G, and H). In summary, the results suggest that under MCD conditions, ferroptosis plays a significant role in the hepatotoxicity induced by the combination of LA-m and LA-l.

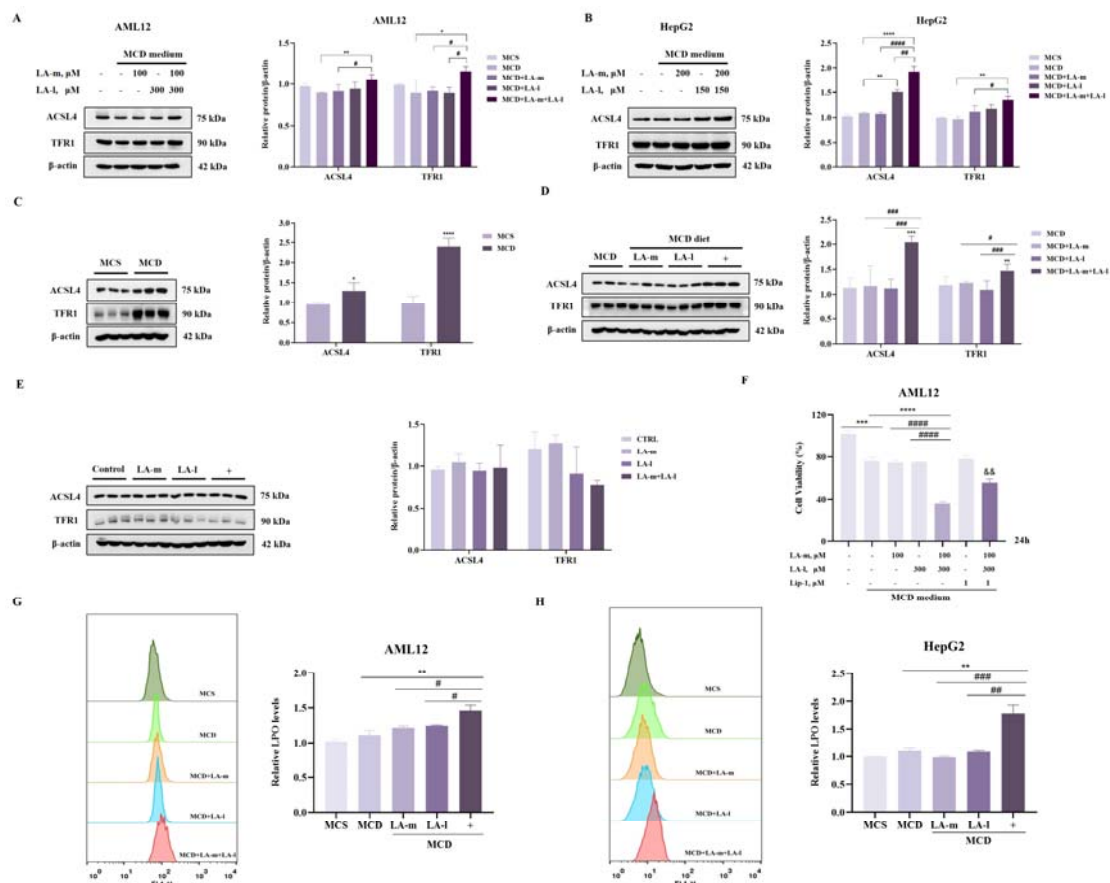


Fig.6 Co-exposure to LA-m and LA-l increases ACSL4 and TFR1 expression *in vitro* and *in vivo* MCD conditions, contributing to exacerbate MASH progression. (A-E) The relative expression and quantitative analysis of ACSL4 and TFR1 were performed in AML12 (A), HepG2 cells (B), MCD diet-fed mice (C and D) and ND-fed mice (E) treated with LA-m and LA-l alone or their combination. (F) AML12 cells were pretreated with 1 μ M Lip-1 for 6 h and then stimulated with LA-m and LA-l alone or their combination for 24 h to assess cell viability. (G and H) LA-m and LA-l combination induced upregulation of LPO levels in MCD medium, quantified in AML12 (G) and HepG2 cells (H) using the Liperfluor kit. Results are indicated as mean $\pm$ SD (n = 3), * P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001 versus the MCS or MCD group. # P < 0.05, ## P < 0.01, ### P < 0.001, and #### P < 0.0001 versus the LA-m or LA-l group. &# P < 0.01 versus the LA-m + LA-l group.

4. Discussion

To establish specific dietary guidelines for patients with metabolic disorders, safety issue is an important factor that needs to be considered. To this end, the potential risk of LA-m and LA-l combination for MASH patients was evaluated using MCD medium-induced in vitro MASH model and MCD diet-induced MASH mouse model. Both models demonstrated that co-exposure of LA-m and LA-l at clinically relevant doses resulted in a significant hepatocyte toxicity and more severe hepatic steatosis in MASH condition but not in healthy metabolic condition. Moreover, even if the experiment duration was extended to 12 weeks, no toxicity was observed in mice fed a normal diet based on the measurement of parameters such as the body weight, food intake, water intake, ALT, AST, Cre, BUN, TG, and organ index of mice (Figure S1A-P). These results implicate a potential risk for MASH patients when co-intake of LA-m and LA-l at comparable levels used in the present study.

The 2005 Dietary Guidelines for Americans Committee recommended 3 cups (720 mL) (494 mg/kg LA-m) of milk and dairy products per day [36]. Dietary Guidelines for Chinese Residents (2022) recommends 300-500 g (200-333 mg/kg LA-m) of milk and dairy products per day. In addition, the Chinese Nutrition Society recommends 25-30 g (315-379 mg/kg LA-l) of fat per day. In fact, literature revealed that the dosage of LA-l used is typically far below the daily dietary recommended intake. For example, in the study by Gu et al., the therapeutic intervention dosage of LA-l, after dietary supplementation conversion, is approximately 100 mg/kg [37]. However, Fang et al. found that in recent years, the fat intake of Chinese residents has been 79.1 g (998 mg/kg LA-l), which is about three times the recommended amount by the Chinese Nutrition Society [38]. In this study, we aim to simulate daily dietary intervention. Therefore, based on the aforementioned literatures, the doses of 350 mg/kg LA-m and 1000 mg/kg LA-l (1:3) used in the present study are clinically relevant.

Sphingolipids are synthesized in the endoplasmic reticulum and dysregulation of sphingolipid metabolism can cause ER stress [39]. Persistent ER stress or adaptive response failure can lead to cell apoptosis [40]. Ceramide serves as a major molecule in sphingolipid metabolism and can be synthesized by *de novo* synthesis, hydrolyzed from sphingomyelin by sphingomyelinase (SMase), and glycosphingolipids catabolism [41]. Previous studies have demonstrated that ceramides enhance membrane thickness and stiffness, which in turn facilitate protein misfolding and aggregation, while inhibiting protein transport. Furthermore, specific ceramide species could induce ER stress [39]. In Yao et al.'s study, disruptions in sphingolipid metabolism and accumulation of ceramides have been shown to trigger ER stress and apoptosis in retinal pigment epithelial cells under UV and hydrogen peroxide exposure [42]. Furthermore, changes in sphingolipid levels can exacerbate ER stress and impair mitochondrial function during the aging process [43]. In this study, lipidomics results showed that under MCD diet feeding, the combination of LA-m and LA-l led to an increase in ceramide and sphingomyelin. To further verify whether sphingolipid-induced ER stress plays a role in hepatotoxicity induced by LA-m and LA-l. We applied with the sphingolipid inhibitor ISP-1 for 6 hours in an MCD medium, and found that it partially reversed the decrease in cell viability induced by the combination of

LA-m and LA-l in HepG2 cells, and downregulated the expression of p-IRE1 α /IRE1 α and p-JNK/JNK. Thus, our experimental results provide evidence that ER stress induced by sphingolipid accumulation was involved in the combination-induced hepatotoxicity under MCD conditions.

In recent years, researchers have uncovered that lipid metabolism holds a crucial role in ferroptosis, a form of non-apoptotic cell death characterized by the peroxidation of membrane lipids. Among various lipids, phospholipids containing PUFAs are particularly significant in triggering ferroptosis through their oxidation [35]. The enzymes involved in lipid metabolism, such as ACSL4 and ACSL3, can regulate the susceptibility to ferroptosis by shifting the balance of PUFA-containing versus monounsaturated fatty acid (MUFA)-containing phospholipids [44]. In our study, we observed a notable increase in ACSL4 protein levels by the combination of LA-m and LA-l in MCD medium cultured hepatocytes and MCD diet-fed mice. Moreover, lipidomics data indicated that the combination of LA-m and LA-l significantly increased the fatty acid side chains containing AA/AdA in PE, which are key membrane phospholipids leading to ferroptosis [35]. Given that linoleic acid serves as a precursor to AA, we speculated that the induction of hepatotoxicity by combining LA-m with LA-l might be attributed to ability of LA-m to enhance the esterification of LA-l via up-regulation of ACSL4, which in turn facilitated ferroptosis. This finding is consistent with the results reported by Han et al [45]. Indeed, our data showed that ferroptosis inhibition by its inhibitor partially protected liver cells from the combination of LA-m and LA-l-induced cytotoxicity, supporting involvement of ferroptosis induction in the hepatotoxicity by co-intake of LA-m and LA-l.

There are a few caveats about our work. We have not addressed the mechanisms involved in LA-m/LA-l-mediated hepatic lipid metabolism dysregulation, particularly, the enzymes targeted by LA-m/LA-l leading to SP accumulation. We speculated that the SP accumulation by LA-m /LA-l might be achieved by enhancing the activity of serine palmitoyltransferase (SPT) or enzymes involved in the hydrolysis of sphingomyelin and the breakdown of glycosphingolipids. In addition, the effects of combining LA-l with other members of MCFAs or mixture of MCFAs on hepatotoxicity under MASH condition will need to be investigated in future studies.

5. Conclusion

In conclusion, combination of LA-m and LA-l aggravated hepatotoxicity in MCD diet-induced MASH mouse model, mechanistically by SP accumulation-mediated ER stress and GP accumulation-driven ferroptosis (Figure 7). The findings provide novel information for establishing dietary guideline for MASH patients.

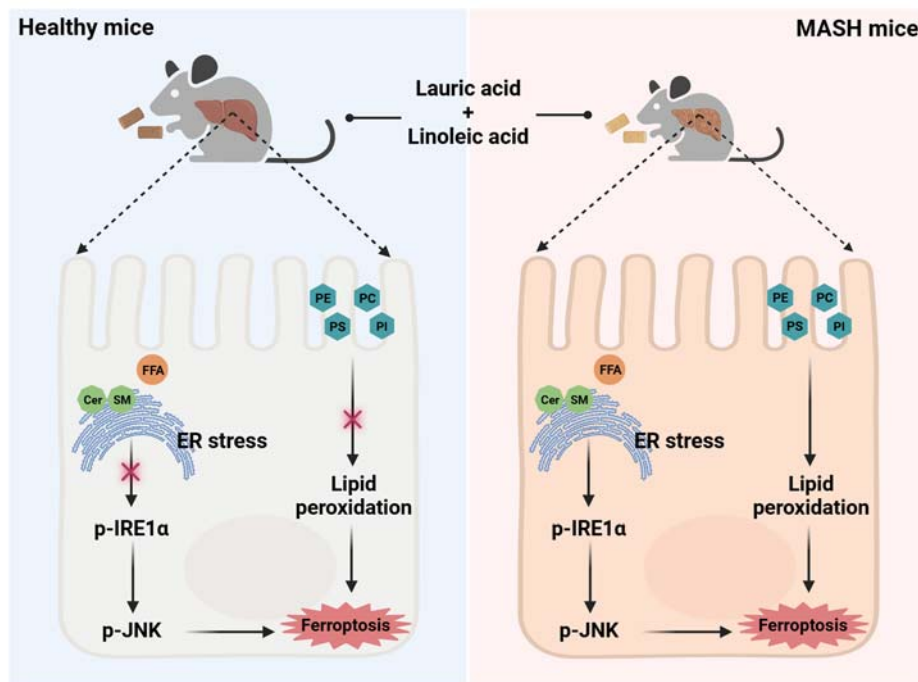


Fig.7 Schematic diagram of co-intake of LA-m and LA-l exacerbates hepatotoxicity in the MASH mice but not in healthy mice. Under MASH condition, LA-m and LA-l combination induces the accumulation of SP, activating the ER stress and ultimately triggering ferroptosis. In addition, LA-m and LA-l combination leads to an increase in GP levels, which drives the ferroptosis.

Conflict of interest

The authors declare no conflicts of interest.

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