Liver Research 8 (2024) 152–164

Contents lists available at ScienceDirect

Liver Research

journal homepage: http://www.keaipublishing.com/en/journals/liver-research

Original Article

Meldonium-induced steatosis is associated with increased delta 6 desaturation and reduced elongation of n-6 polyunsaturated fatty acids

Bodil Bjørndal [a, b], Siri Lunde Tungland [c], Pavol Bohov [a], Magne O. Sydnes [c], Simon N. Dankel [a], Lise Madsen [d, *], Rolf K Berge [a, e, **]

[a] Department of Clinical Science, University of Bergen, Bergen, Norway
[b] Department of Sports, Physical Activity and Food, Western Norway University of Applied Sciences, Bergen, Norway
[c] Department of Chemistry, Bioscience and Environmental Engineering, University of Stavanger, Stavanger, Norway
[d] Department of Clinical Medicine, University of Bergen, Bergen, Norway
[e] Department of Heart Disease, Haukeland University Hospital, Bergen, Norway

ARTICLE INFO

Article history:
Received 25 January 2024
Received in revised form
24 June 2024
Accepted 3 September 2024

Keywords:
Steatosis
Fatty acid composition
Fatty acid oxidation
Meldonium
Desaturase
Elongase
n-6 polyunsaturated fatty acid

ABSTRACT

Background and objective: Metabolic associated fatty liver disease (MAFLD) is associated with abnormal lipid metabolism. Mitochondrial dysfunction is considered an important factor in the onset of MAFLD, whereas altered fatty acid composition has been linked to the severity of the disease. Tetradecylthioacetic acid (TTA), shown to induce mitochondrial proliferation and alter the fatty acid composition, was used to delay the accumulation of hepatic triacylglycerol. This study aimed to evaluate how impaired mitochondrial fatty acid beta-oxidation affects fatty acid composition by incorporating meldonium into a high-carbohydrate diet.

Methods: C57BL/6 mice ($n = 40$) were fed high-carbohydrate diets supplemented with meldonium, TTA, or a combination of meldonium and TTA for 21 days. Lipid levels were determined in liver samples, and fatty acid composition was measured in both liver and plasma samples. Additionally, desaturase and elongase activities were estimated. The hepatic activities and gene expression levels of enzymes involved in fatty acid metabolism were measured in liver samples, whereas carnitines, their precursors, and acylcarnitines were measured in plasma samples.

Results: The meldonium-induced depletion of L-carnitine and mitochondrial fatty acid oxidation was confirmed by reduced plasma levels of L-carnitine and acylcarnitines. Principal component analyses of the hepatic fatty acid composition revealed clustering dependent on meldonium and TTA. The meldonium-induced increase in hepatic triacylglycerol levels correlated negatively with estimated activities of elongases and was associated with higher estimated activities of delta-6 desaturase (D6D; C18:4n-3/C18:3n-3 and C18:3n-6/C18:2n-6), and increased circulating levels of C18:4n-3 and C18:3n-6 (gamma-linolenic acid). TTA mitigated meldonium-induced triacylglycerol levels by 80% and attenuated the estimated D6D activities, and elongation of n-6 polyunsaturated fatty acids (PUFAs). TTA also attenuated the meldonium-mediated reduction of C24:1n-9 (nervonic acid), possibly by stimulating *Elovl5* and increased elongation of erucic acid (C22:1n-9) to nervonic acid. The hepatic levels of nervonic acid and the estimated activity of n-6 PUFA elongation correlated negatively with the hepatic triacylglycerol levels, while the estimated activities of D6D correlated positively.

Conclusion: Circulating levels of gamma-linolenic acid, along with reduced estimated elongation of n-6 PUFAs and D6D desaturation activities, were associated with hepatic triacylglycerol levels.

* Corresponding author. Department of Clinical Medicine, University of Bergen, Bergen, Norway.
** Corresponding author. Department of Clinical Science, University of Bergen and Department of Heart Disease, Haukeland University Hospital, Bergen, Norway.
E-mail addresses: lise.madsen@uib.no (Lise Madsen), rolf.berge@uib.no (Rolf K Berge).

https://doi.org/10.1016/j.livres.2024.09.001

1. Introduction

Metabolic associated fatty liver disease (MAFLD) is characterized by the accumulation of fat in the liver and encompasses a wide spectrum of liver damage, ranging from simple hepatic fat accumulation to steatohepatitis, termed non-alcoholic steatohepatitis (NASH) or metabolic dysfunction-associated steatohepatitis (MASH), progressive fibrosis, and cirrhosis.[1] MAFLD is one of the most common chronic liver diseases, with a global prevalence approaching 30% and showing a rising trend.[2] Although there were previously no approved pharmacological therapies for MAFLD,[3] in 2024, Resmetirom, a liver-directed, thyroid hormone receptor beta-selective agonist,[4] did show promise in clinical trials for treating non-cirrhotic MAFLD with moderate to advanced fibrosis and was approved by the U.S. Food and Drug Administration.[5] However, since the simple accumulation of liver fat is a risk factor for progression to steatosis and liver failure, identifying early-stage dysregulation of fatty acid (FA) metabolism can be an early biomarker of liver pathology and promote treatment at earlier stages of the disease.

MAFLD is closely associated with metabolic dysfunction and the term is therefore more appropriate than the earlier used term non-alcoholic fatty liver disease.[6] In particular, mitochondrial dysfunction is reported to play a central role in both the onset and progression of the disease.[7] It has been observed in patients with fatty liver disease and has been linked to increasing MAFLD severity in patients with obesity.[8,9] Furthermore, mitochondrial dysfunction is associated with reduced mitochondrial FA β-oxidation in MAFLD patients and the induction of MAFLD in mice.[9–12] Mitochondrial FA β-oxidation requires carnitine for the transport of long-chain FAs into the mitochondria. As the carnitine shuttle is rate-limiting, hepatic carnitine concentrations regulate the rate of mitochondrial FA β-oxidation.[13] Additionally, L-carnitine biosynthesis, and hence, L-carnitine levels and mitochondrial FA β-oxidation capacity, can be reduced by the anti-ischemic drug meldonium (also known as mildronate),[14,15] and meldonium-induces hepatic steatosis in rats.[16,17]

Fat accumulation in the liver results from an imbalance between FA uptake, synthesis, and disposal. Hence, together with reduced mitochondrial FA β-oxidation and potentially increased hepatic FA synthesis, the increased delivery and transport of free FAs into the liver are implicated in the pathogenesis of MAFLD. Free FAs released from adipose tissue through lipolysis are the major source of triacylglycerol (TAG) stored in the liver,[18] and the levels of circulating free FAs have been associated with the severity of steatosis.[19–21] Additionally, plasma levels of certain FAs, such as palmitoleic acid (C16:1n-7), vaccenic acid (C18:1n-7),[22] as well as myristic acid (C14:0), are suggested as potential predictors of MAFLD severity.[23] Notably, in male Wistar rats, the ratio of fat-to-carbohydrate intake was also found to influence fatty liver and associated FA composition in plasma and liver, which appeared to involve altered hepatic lipogenic enzyme activities rather than reduced FA β-oxidation.[24] Moreover, altered activities of elongases and desaturates have been associated with the severity of MAFLD and the development of MASH. For instance, Walle *et al.*[19] reported a higher hepatic 16:1n-7/16:0 ratio in MASH, reported as NASH patients, indicating increased delta-9 desaturation (D9D), and a lower 20:4n-6/20:3n-6 ratio, indicating decreased D5D. A high hepatic C16:1n-7/C16:0 ratio combined with a high C18:1n-9/C18:0 ratio, and a low C18:0/C16:0 ratio, has also been associated with the progression from simple steatosis to NASH or MASH, as indicated by Yamada *et al.*[25] Furthermore, the C16:1n-7/C16:0 ratio in both serum and liver tissue has been correlated with the progression of MASH.[26]

It is unclear why FA composition in the liver and blood is related to MASH progression, but the reported changes in FA ratio indices suggest that the progression of severity may involve alterations in hepatic D5D, D6D, and D9D activities, as well as elongase activity and *de novo* FA synthesis.[19,24,26,27] Given the potential significance of mitochondrial function in the onset of the disease, we aimed to evaluate how reduced mitochondrial FA β-oxidation affects the FA composition, as well as estimated elongase and desaturase activities, by incorporating meldonium into a high-carbohydrate diet. Tetradecylthioacetic acid (TTA) is a structurally modified FA that cannot undergo β-oxidation due to the sulfur atom at the 3-position of the carbon chain. It is known to be a pan-peroxisome proliferator-activated receptor-ligand, and TTA treatment reduces the levels of TAGs in the liver by inducing mitochondrial proliferation and mitochondrial β-oxidation capacity. Additionally, TTA is recognized for inducing changes in FA composition and desaturase activities.[28–32] We herein demonstrate that TTA attenuates meldonium-induced steatoses and restores the changes in FA desaturation and elongation induced by meldonium.

2. Materials and methods

2.1. Ethics approval

The animal study was conducted in accordance with the ethical guidelines for the use of animals in research, as governed by the Norwegian Regulations Relating to the Use of Animals in Research, which follow the Animal Welfare Act. The protocol for animal experiments was ethically reviewed and approved by the National Animal Research Authority (Project no. FOTS 5071).

2.2. Animals and treatment

Upon arrival, 10-week-old male C57BL/6 J mice (Taconic Biosciences, Ejby, Denmark) were randomized into Makrolon III cages, with 2 animals per cage, in an open system under standard laboratory conditions. The environment was maintained at a temperature of 22 ± 1 °C, with dark/light cycles of 12/12 h, relative humidity 55 ± 5%, and 20 air changes per hour. The animals had free access to tap water and feed.

Cages were randomized into 4 groups of 10 mice determined by the start/killing day and rack placement (www.random.org). After 7 days of acclimatization, all groups were given low-fat diets containing 16% energy from fat, 64% energy from carbohydrates, and 20% energy from protein, supplemented with or without meldonium, kindly provided by Grindex (Riga, Latvia) and/or TTA (Table 1). Based on average feed intake, the supplemented amount of meldonium corresponds to 550 mg meldonium/kg body weight/day, a dose earlier demonstrated to induce steatosis in mice.[17] The amount of TTA corresponds to a dose of 720 mg of TTA/kg body weight/day, a dose previously demonstrated to modify hepatic lipid metabolism in mice.[33] All mouse groups received the same basic diet, similar to the Basal diet 5755, with adequate essential FA content (1.6 g C18:2n-6, 0.15 g C18:3n-3 per 100 g of diet) and only traces of their long-chain polyunsaturated FA (PUFA) metabolites (0.01 g C20:4n-6, 0.001 g C20:5n-3, and 0.003 g C22:6n-3 per 100 g of diet). The remaining individual long-chain PUFAs ranged from 0.040 to 0.001 g per 100 g of diet. Thus, the lipid effect of diet should be the same in all groups. The only dietary differences were the added drugs. Feed was provided to the mice in a fixed daily amount, and leftover feed was removed and weighed daily during the 21-day experiment. Weight gain was measured twice a week. All animals were alive at the end of the experiment.

At the time of sacrifice, mice were anesthetized by inhalation of 2%–5% sevoflurane (Abbott Laboratories Ltd., Berkshire, UK) after fasting for 4 h. Blood was collected from the right ventricle of the heart into a tube containing 7.5% EDTA. The samples were chilled on

Table 1
Composition of the experimental diet.

Components	g/kg
Carbohydrates	**679**
Cornstarch	397
Dextrose	132
Sucrose	100
Cellulose fiber	50
Protein	**225**
Casein[a]	225
Fat	**70**
Soybean oil	20
Lard	50
Micronutrients	**50.5**
AIN-93G-MX mineral mix	35
AIN-93G-VX vitamin mix	10
L-cysteine	3
Choline bitartrate	2.5
tert-Butylhydroquinone	0.014
Drugs [b]	
Meldonium (0.306%)	3.06
Tetradecylthioacetic acid (0.4%)	4.0

[a] The protein account in casein was 87%, the rest was moisture, ash, and small amounts of fat and lactose.
[b] The drugs were added to their respective intervention groups.

ice for about 15 min after which plasma was separated by centrifugation and stored at −80 °C until further analysis. Plasma samples were pooled from 2 to 3 animals to obtain a sufficient volume of plasma for the analyses described in section 2.3 ($n = 4 - 6$). The liver was harvested and stored at −80 °C until further processing.

2.3. Liver and plasma FA composition and lipid levels

Lipids were extracted from pooled plasma samples and 8 randomly selected livers per group according to the Bligh and Dyer method,[34] evaporated under nitrogen and redissolved in isopropanol before analysis. FA methyl esters were obtained by heating the lipids with methanol at 90 °C for 1 h in the presence of sulphuric acid and analyzed by gas-liquid chromatography (GC-8000 TOP, Finnigan, USA), as previously described.[35] Hepatic lipids were also quantified enzymatically on a Hitachi 917 system (Roche Diagnostics GmbH, Mannheim, Germany) using the triacylglycerol (GPO-PAP) and cholesterol kit (CHOD-PAP) from Roche Diagnostics (Roche Diagnostics International Ltd, Rotkreuz, Switzerland), and the phospholipids FS kit from DiaSys Diagnostic Systems GmbH (Holzheim, Germany).

2.4. Liver enzyme activities

Post-nuclear fractions were prepared by homogenizing 100 mg liver in 1 mL ice-cold sucrose medium (0.25 mol/L sucrose, 10 mmol/L HEPES (pH 7.4), and 2 mmol/L EDTA as earlier described.[36] Palmitoyl-coenzyme A (palmitoyl-CoA) oxidation was measured in the post-nuclear fraction as the production of acid-soluble products from [1−^{14}C] palmitoyl-CoA in the presence of 1.2 mmol/L L-carnitine as described.[37] The post-nuclear fraction was frozen at −80 °C and later used to measure the activity of acyl-CoA oxidase 1, palmitoyl (ACOX1) using a coupled assay based on the hydrogen peroxide-dependent oxidation of leuco-dichlorofluorescein in the presence of palmitoyl-CoA.[38] The production of hydrogen peroxide was measured by monitoring the increase in dichlorofluorescein absorbance using a Varian 23,000 spectrophotometer. Carnitine palmitoyl transferase 2 (CPT2) capacity was measured after lysis with 0.01% Triton-X using labeled [methyl-^{14}C]-L-carnitine, as described by Bremer with modifications described by Madsen and Berge and FA synthesis activity was measured using [^{14}C] acetyl-CoA as described.[39–41]

2.5. Gene expression analysis

Total cellular RNA was purified from the same liver samples selected for FA composition (Section 2.3), and complementary DNA was generated as previously described.[24] Real-time polymerase chain reaction (PCR) was performed with Sarstedt 384 well multiply-PCR plates (Sarstedt Inc., Newton, NC, USA). The following genes were measured: *Cpt2*, *Acox1*, stearoyl-CoA desaturase 1 (*Scd1*), fatty acid desaturase 2 (*Fads2*), and fatty acid elongase 5 (*Elovl5*) using probes and primers from Applied Biosystems (Foster City, CA, USA). Three different reference genes were used:18S ribosomal RNA ((*18S*, Kit-FAM-TAMRA (Reference RT-CKFT-18s) from Eurogentec, Seraing, Belgium), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, Mm99999915_g1, from Applied Biosystems, Foster City, CA, USA), ribosomal protein lateral stalk subunit P0 (*Rplp0*, Gene ID 11837, from Thermo Fisher (Fisher Scientific GmBH, Schwerte, Germany). NormFinder was used to assess the optimal reference genes, and data normalized to *Rplp0* were used (https://moma.dk/normfinder-software).

2.6. Measurements of plasma levels of carnitines, their precursors, and acylcarnitines

Plasma levels of L-carnitine, its biosynthetic precursor trimethyllysine, and gamma-butyrobetaine, as well as short- and long-chain acylcarnitines, were analyzed in pooled samples using high-performance liquid chromatography/mass spectrometry, as described by Vernez *et al.*,[42] with some modifications of the high-performance liquid chromatography conditions as described in detail by Vigerust *et al.*[43]

2.7. Statistical analyses

Data sets were analyzed using Prism Software (GraphPad Prism 9.0.0, San Diego, CA, USA). Unless otherwise stated in the figure legends, results are presented as means of 8 animals per group. The Shapiro-Wilk test was used to estimate the normal distribution, and one-way ANOVA with Tukey's post hoc test was used to determine statistical significance among the feeding groups. Two-tailed P values < 0.05 were considered significant. Pearson correlation coefficients were determined with two-tailed P values and a 95% confidence interval.

3. Results

3.1. TTA attenuates meldonium-induced steatosis

To verify meldonium-induced steatosis and attenuation by TTA, total levels of TAGs, lipids, FAs, phospholipids, and cholesterol were measured in the liver. The relative liver weight (hepatic index) was also determined. Carnitine depletion from meldonium treatment did not significantly increase the hepatic index (Fig. 1A), but it doubled the accumulation of total fat in the liver (Fig. 1B). As described earlier,[33] TTA led to an increased hepatic index (Fig. 1A). As expected, the inclusion of TTA strongly attenuated meldonium-induced fat accumulation measured as total lipid (Fig. 1B) and total FAs (Fig. 1C). Hepatic TAG levels reflected the levels of total fat, although there was an even greater increase by meldonium (Fig. 1D), whereas the levels of phospholipids and cholesterol were not significantly increased by meldonium (Fig. 1E–F). Hence, TTA treatment can counteract meldonium-induced steatosis in mice fed a high-carbohydrate diet.

3.2. TTA attenuates meldonium-induced reduction in plasma concentrations of carnitine and acylcarnitines and stimulates peroxisomal β-oxidation

Meldonium treatment prevents carnitine biosynthesis by inhibiting the formation of its precursor γ-butyrobetaine from trimethyllysine.[44] In line with this, meldonium treatment led to reduced plasma levels of γ-butyrobetaine and L-carnitine, whereas the precursor trimethyllysine levels were unchanged (Fig. 2A). Meldonium treatment inhibits mitochondrial FA oxidation, as L-carnitine is required for FAs to enter mitochondria.[13] In line with this, we observed reduced plasma levels of acylcarnitine, including palmitoylcarnitine, in meldonium-treated mice (Fig. 2A), whereas the hepatic capacity to convert palmitoyl-CoA to acid-soluble products *ex vivo* in a L-carnitine supplemented liver homogenate was not inhibited (Fig. 2A).

TTA is known to target mitochondria, and it has been previously demonstrated that TTA-mediated stimulation of mitochondrial β-oxidation is associated with increased levels of plasma acylcarnitines.[33] In line with this, plasma acylcarnitine levels were significantly increased in TTA-treated mice (Fig. 2A). TTA was not able to rescue the meldonium-mediated decrease in neither γ-butyrobetaine, L-carnitine nor acylcarnitine levels (Fig. 2A). However, the hepatic capacity to convert palmitoyl-CoA to acid-soluble products *ex vivo* in an L-carnitine supplemented liver homogenate was increased in mice treated with meldonium and TTA in combination (Fig. 2B). In line with this, CPT2 capacity was increased in a similar manner (Fig. 2B).

Peroxisomal β-oxidation does not require L-carnitine, increased peroxisomal β-oxidation may occur in meldonium-treated mice as observed in meldonium-treated rats.[45] We measured the activity and gene expression of acyl-CoA oxidase 1, palmitoyl (ACOX), the rate-limiting enzyme of peroxisomal β-oxidation. Both the ACOX activity and *Acox1* mRNA expression were increased in meldonium and TTA- treated mice (Fig. 2C). Hence, FAs may be shortened by peroxisomal β-oxidation in the presence of meldonium.

3.3. TTA attenuates meldonium-induced changes in hepatic FA composition

To investigate the meldonium-induced changes in FA metabolism, we measured FA composition. Principal component analyses of the FA composition demonstrated a clear separation of the groups, with PC1 and PC2 explaining 39% and 32%, respectively of the variance in the liver (Fig. 3A). Samples from mice treated with or without meldonium are separated along PC1, whereas samples from mice treated with or without TTA are separated along PC2. n-3 and n-6 FAs with chain lengths C18 and C20, as well as C24:1n-9, were the main contributors to separation along PC1, whereas saturated FAs (SFAs), including C20:0 and C23:0 were the main contributors to separation along PC2. We next calculated the estimated elongase and desaturase activities and prepared a heat map

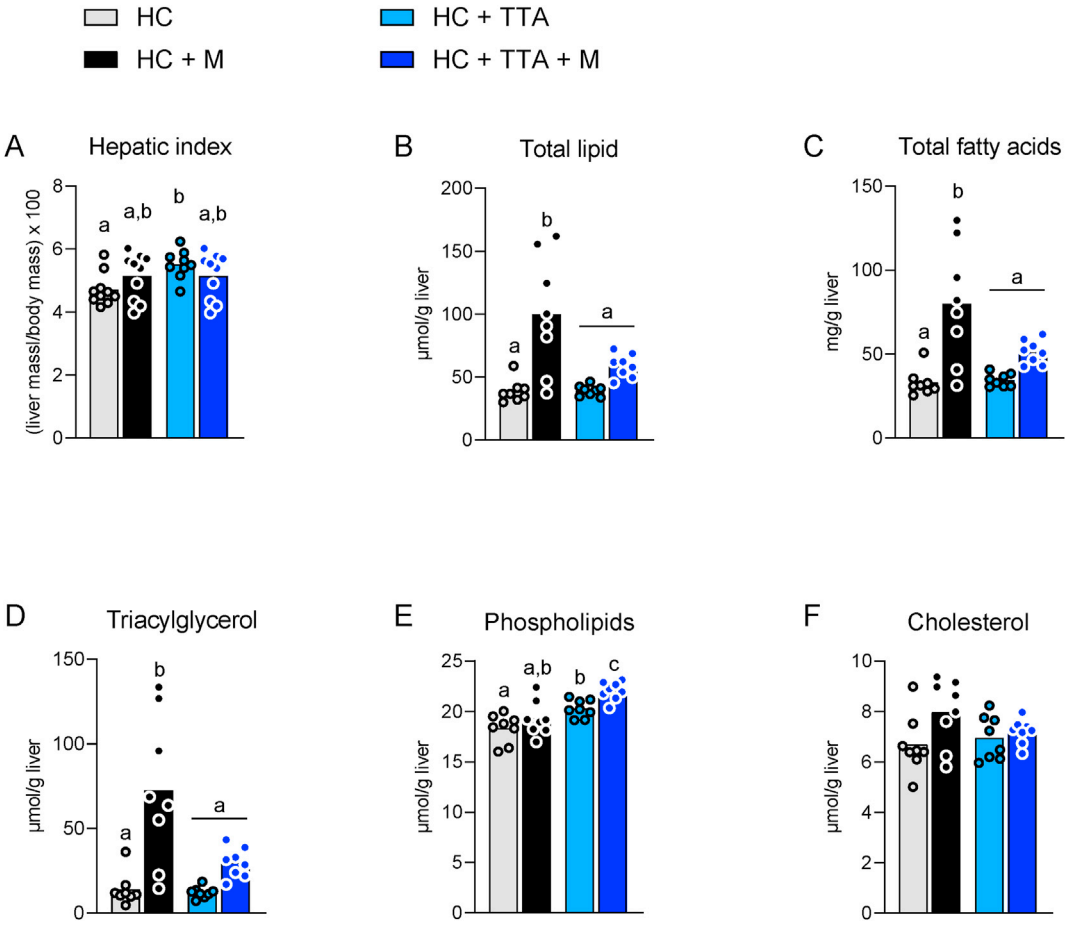

Fig. 1. Hepatic lipid levels. Hepatic index **(A)**, and hepatic levels of total lipid **(B)**, total fatty acids extracted from all lipid classes **(C)**, triacylglycerol **(D)**, phospholipids **(E)**, and cholesterol **(F)** were measured in C57BL/6 mice fed a high-carbohydrate control diet (HC) or a carbohydrate diet spiked with meldonium (HC + M), tetradecylthioacetic acid (HC + TTA), or TTA and meldonium (HC + TTA + M) for 21 days. Total liver lipids were calculated as the sum of individual lipid fractions. The bars represent the mean of the individual values shown as dots. One-way ANOVA with Tukey's post hoc test was used to determine significant differences between the intervention groups. Different letters indicate statistical significance ($P < 0.05$) between groups.

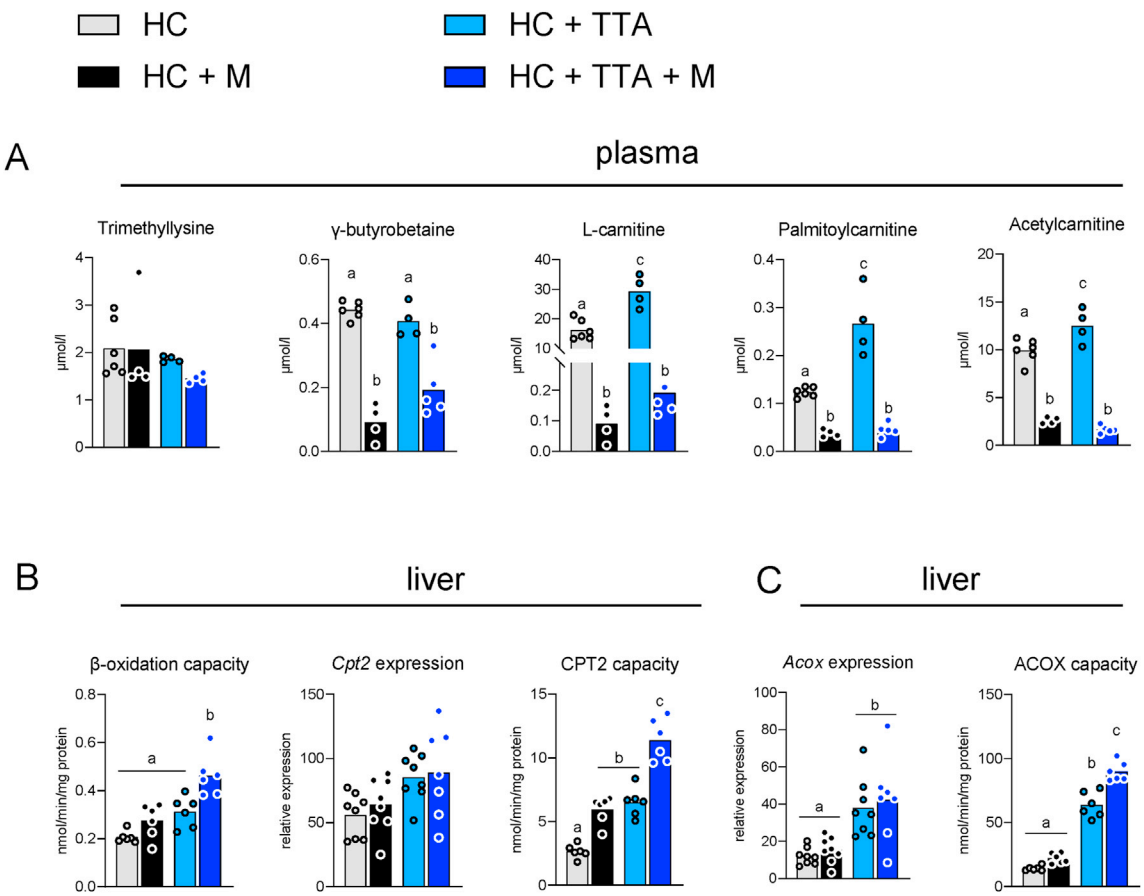

Fig. 2. Plasma carnitine metabolites and fatty acid oxidation capacity. Plasma metabolites **(A)**, hepatic β-oxidation capacity in presence of carnitine and hepatic mitochondrial carnitine palmitoyl transferase 2 (CPT2) expression and capacity **(B)**, and hepatic peroxisomal acyl-CoA oxidase (ACOX) expression and activity **(C)** were measured in C57BL/6 mice given a high carbohydrate control diet (HC) or a carbohydrate diet spiked with meldonium (HC + M), tetradecylthioacetic acid (HC + TTA), or TTA and meldonium (HC + TTA + M) for 21 days. The bars represent the mean of the individual values shown as dots. One-way ANOVA with Tukey's post hoc test was used to determine significant differences between the intervention groups. Different letters indicate statistical significance ($P < 0.05$) between groups. Abbreviations: FAs, fatty acids; TTA, tetradecylthioacetic acid.

to visualize how meldonium treatment affected FA composition and estimated desaturase and elongase activities (Fig. 3B). Most noticeably, meldonium led to a higher proportion of C18 FAs, as well as estimated D6D activities, whereas TTA led to a higher proportion of n-3 and n-6 C20 FAs and higher estimated elongase activities. Hence, TTA may mediate its effect on preventing steatosis by attenuating meldonium-induced changes in FA desaturation and elongation.

3.4. Meldonium-induced steatosis is associated with increased D6D of FA

To investigate the possible association between FA desaturation and steatosis, we prepared a correlation matrix using the hepatic TAG levels, the estimated D5D, D6D, and D9D activities, and associated FAs. The hepatic TAG levels correlated with the estimated C16 D9D activity calculated as the C16:1n-7/C16:0 ratio (D9D C16, $r = 0.685$, $P < 0.0001$) and the C18 D9D activity calculated as the C18:1n-9/C18:0 ratio (C18 D9D, $r = 0.895$, $P < 0.0001$) (Fig. 4A). As expected, C18 D9D correlated positively with C18:1n-9 and negatively with C18:0. C16 D9D correlated positively with C16:1n-7, but no correlation between C16 D9D and C16:0 was found. The hepatic C18:1n-9/C18:0 ratio, but not the C16:1n-7/C16:0 ratio was significantly increased with meldonium (Fig. 4B). TTA treatment increased the hepatic expression of *Scd1*, but neither the hepatic C18:1n-9/C18:0 ratio nor the C16:1n-7/C16:0 ratio was increased by TTA. Of note, TTA treatment led to an increased C18:1n-9/C18:0 ratio in plasma, but the estimated D9D activities in plasma did not correspond to those for the liver. Taken together, increased D9D C18 activity may be associated with steatosis, but it is not likely that TTA mediates its action by inhibiting D9D.

The estimated D6D activities, calculated as C18:4n-3/C18:3n-3 (n-3 D6D) and C18:3n-6/C18:2n-6 (n-6 D6D) also correlated with hepatic TAG levels ($r = 0.756$, $P < 0.0001$ and $r = 0.779$, $P < 0.0001$, respectively). The estimated D6D n-3 and n-6 activities correlated with each other, as well as with the relative amounts of C18n-3 and C18n-6 FAs (Fig. 4A), suggesting a coordinated regulation. Although TTA treatment induced expression of *Fads2* encoding D6D, mice treated with TTA did not have higher estimated D6D activity (Fig. 4C). Still, the meldonium-induced increases in estimated D6D activities were attenuated by the inclusion of TTA in the diet (Fig. 4C). Hence, estimated D6D activities may be markers of steatosis. Worth noting, plasma C18:4n-3/C18:3n-3 and C18:3n-6/C18:2n-6 ratios reflected the hepatic ratios (Fig. 4C). Taken together, increased D6D is associated with hepatic steatosis, and TTA may in part attenuate steatosis by attenuating the meldonium-induced D6D.

3.5. Meldonium-induced steatosis is associated with reduced FA synthesis and elongation of FAs

To investigate the possible association between meldonium-induced steatosis and changes in FA synthesis and elongation, we

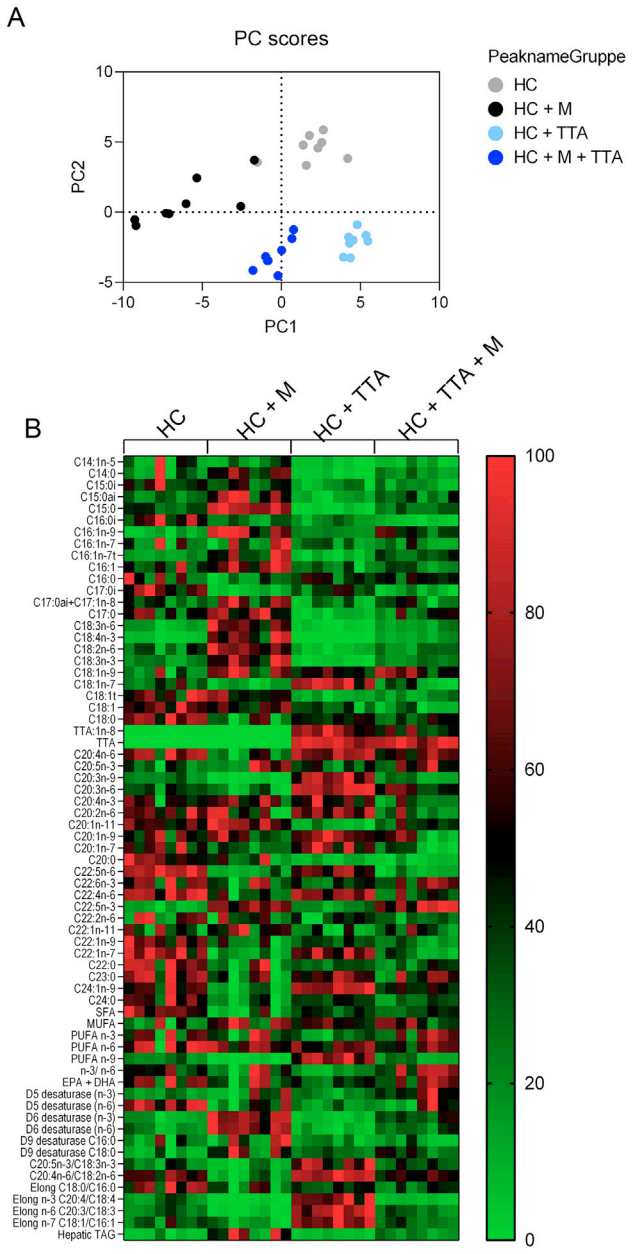

Fig. 3. The relative fatty acid composition in the liver. Principal component analyses score of fatty acids (FAs) extracted from liver **(A)** from C57BL/6 mice given a high-carbohydrate control diet (HC) or a high-carbohydrate diet spiked with meldonium (HC + M), tetradecylthioacetic acid (HC + TTA), or meldonium + TTA (HC + M + TTA) for 21 days. The heatmaps **(B)** illustrate the relative levels of individual FAs, the sum of saturated FAs (SFAs), monounsaturated FAs (MUFAs), n-3 and n-6 polyunsaturated FAs (PUFAs), eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA), the anti-inflammatory index, estimated elongase and desaturase activities in the liver. The data set includes data from 8 animals in each group. Abbreviations: ai (anteiso), i (iso), and t (trans).

prepared a correlation matrix using the hepatic TAG levels, the estimated *de novo* FA synthesis, and elongation activities, as well as the relative levels of their substrates and products. Surprisingly, the estimated *de novo* FA synthesis correlated negatively with the hepatic TAG levels ($r = -0.545$, $P < 0.005$) (Fig. 5A), and meldonium treatment led to decreased FA synthesis measured as the hepatic C16:0/C18:2n-6 ratio as well as FA synthesis activity, measured *ex vivo* (Fig. 5B). These data indicate that increased FA synthesis does not participate in meldonium-induced steatosis.

Elongation of SFAs also correlated negatively with hepatic TAG levels ($r = -0.667$, $P < 0.0001$) (Fig. 5A), but TTA supplementation did not significantly restore the meldonium-mediated reduction in elongation of SFAs or the activity of FA synthesis (Fig. 5B). The estimated activities of elongases targeting n-3, n-6, and n-7 FAs estimated by the C20:4n-3/C18:4n-3, C20:3n-6/C18:3n-6, and C18:1n-7/C16:1n-7 ratios, respectively, all correlated positively with each other and negatively with the hepatic TAG levels ($r = -0.483$, $P < 0.01$, $r = -0.544$, $P < 0.005$, and $r = -0.585$, $P < 0.0005$, respectively) (Fig. 5A). TTA treatment led to higher estimated n-3, n-6, and n-7 elongase activities in the liver and plasma, compared to the control (Fig. 5C). Given the strong correlation between the C20:4n-3/C18:4n-3, C20:3n-6/C18:3n-6, and C18:1n-7/C16:1n-7 ratios and hepatic TAG levels, the estimated elongase activities may be markers of steatosis. However, only the meldonium-induced decrease in estimated n-6 elongase activity was restored by the inclusion of TTA (Fig. 5C).

3.6. Increased circulating levels of C18:4n-3 and C18:3n-6 are associated with meldonium-induced steatosis

We next correlated fat accumulation in the liver with the relative proportion of individual FAs (Fig. 6A). Given the positive association between hepatic TAG levels and D6D activities estimated as C18:4n-3/C18:3n-3 and C18:3n-6/C18:2n-6, it was surprising that the relative proportions of all C18:4n-3, C18:3n-3, C18:3n-6, and C18:2n-6 positively correlated with hepatic TAG (Fig. 6A). Inclusion of TTA attenuated meldonium-induced increase in the relative proportions of all these FAs in the liver (Fig. 6B). The relative proportion of the products of D6D activities, C18:4n-3 and C18:3n-6 (γ-linolenic acid), followed a similar pattern in plasma (Fig. 6B).

In line with the negative association between plasma TAG and elongation of n-6 FAs estimated as C20:3n-6/C18:3n-6, the relative proportion of C20:3n-6 correlated negatively with hepatic TAG, whereas C18:3n-6 correlated positively (Fig. 6A). Meldonium treatment led to a higher proportion of C18:3n-6 in both liver and plasma, and this increase was attenuated by the inclusion of TTA (Fig. 6B), however, the proportion of C20:3n-6 was not reduced by meldonium in either liver or plasma (Fig. 6C). Hence, increased relative proportions of C18n-3 and n-6 PUFAs are associated with hepatic TAG, and the relative proportion of C18:4n-3 and C18:3n-6 in plasma may be possible candidate markers for hepatic steatosis.

We found strong negative correlations between hepatic TAG and the hepatic proportion of C20:4n-6 (arachidonic acid) and C24:1n-9 (nervonic acid) (Fig. 6A). The relative proportion of C20:4n-6 was significantly reduced by meldonium in the liver, and this proportion was restored with TTA (Fig. 6C). However, similar changes were not observed in plasma. The hepatic proportion of C24:1n-9 was also significantly reduced by meldonium (Fig. 6D). The levels were induced by TTA, which attenuated in meldonium-induced reduction (Fig. 6D). C24:1n-9 is proposed to be produced by the elongation of 22:1n-9 (erucic acid), and in line with this, TTA led to a higher C24:1n-9/C22:1n-9 ratio and induced the hepatic expression of *Elovl5* encoding FA elongase 5 that elongates very long-chain FAs. Meldonium treatment did not reduce the abundance of C24:1n-9 in plasma, but the relative proportion was induced by TTA. Hence, increased hepatic TAG is associated with reduced levels of C24:1n-9, and TTA may, at least in part, mediate its action by stimulating elongation of 22:1n-9 to C24:1n-9.

Overall, Fig. 7 presents the experimental model and a summary of the results.

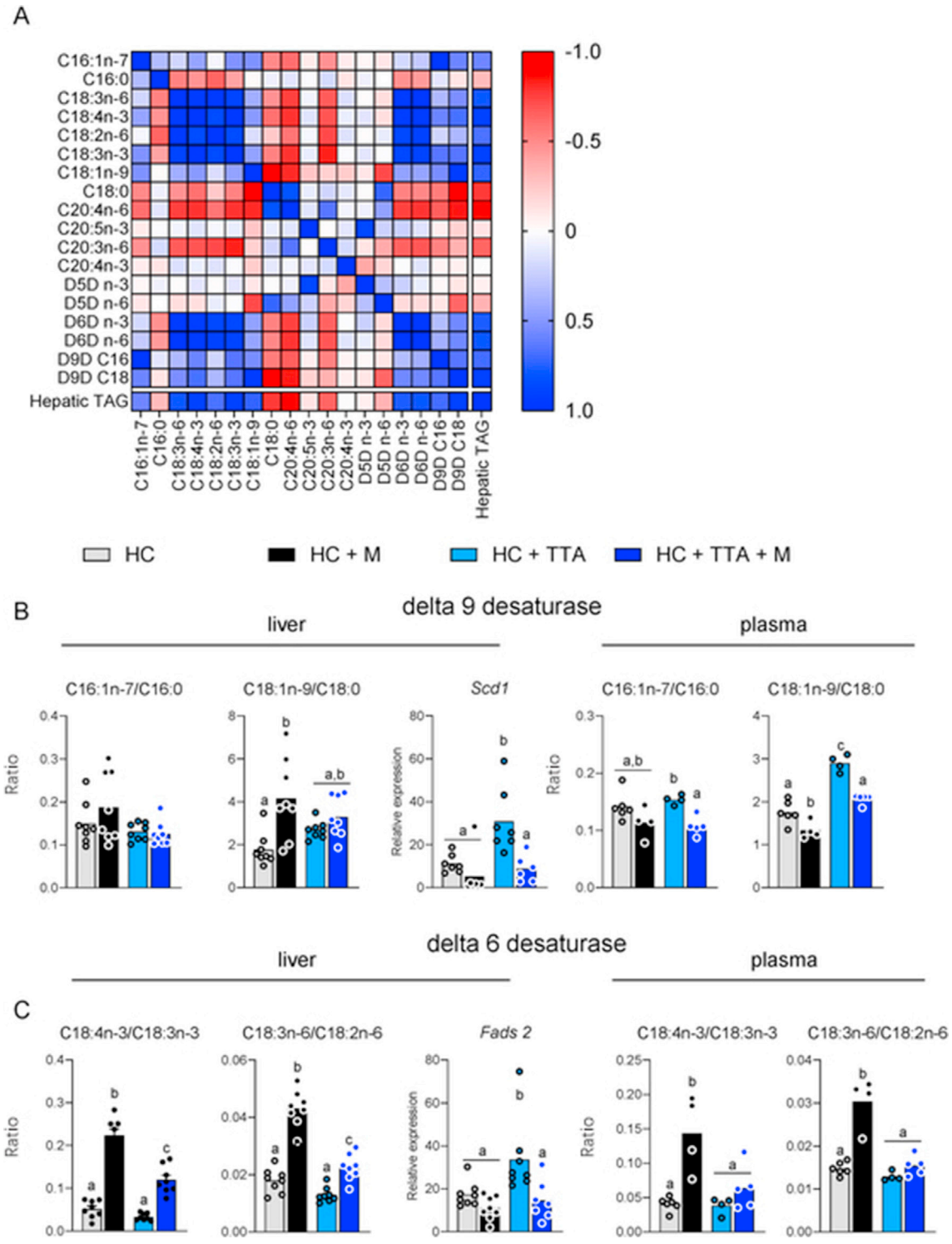

Fig. 4. Estimated delta 9 and delta 6 desaturase activities. Correlation matrix **(A)** of hepatic triacylglycerol levels (TAG) and estimated activities of delta 5 desaturase (D5D), D6D, and D9D activities based on the abundance of fatty acids extracted from the liver of C57BL/6 mice given a high-carbohydrate control diet (HC) or a high-carbohydrate diet spiked with meldonium (HC + M), tetradecylthioacetic acid (HC + TTA) or meldonium and TTA (HC + M + TTA) for 21 days. The bar charts **(B)** illustrate the estimated D9D activities based on the abundance of fatty acids extracted from individual liver and pooled plasma samples and the hepatic relative expression of *Scd1*. The bar charts **(C)** illustrate the delta estimated D6D activities based on the abundance of fatty acids extracted from individual liver and pooled plasma samples and the hepatic relative expression of *Fads2*. The bars represent the mean of the individual values shown as dots. One-way ANOVA with Tukey's post hoc test was used to determine significant differences between the intervention groups. Different letters indicate statistical significance ($P < 0.05$) among groups.

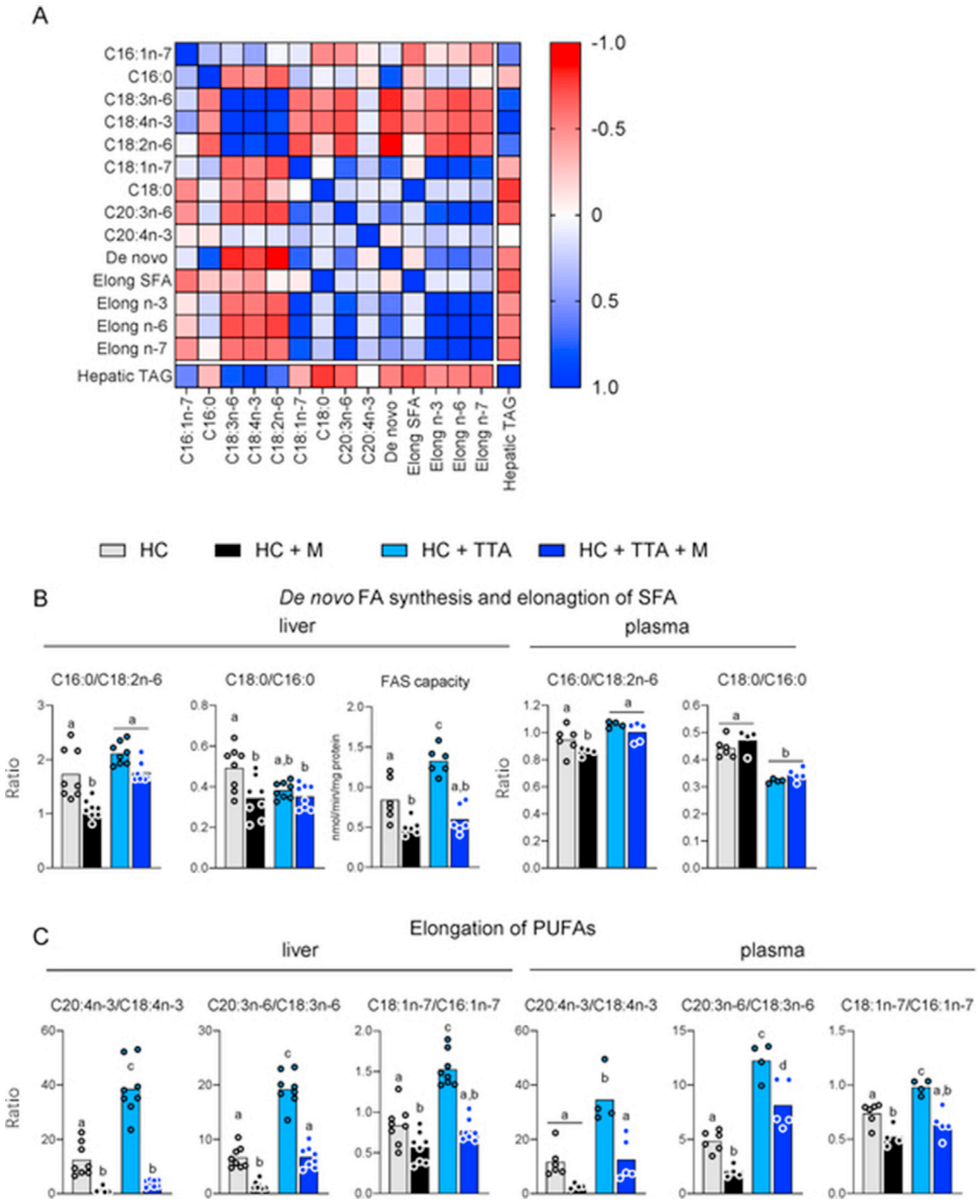

Fig. 5. Estimated *de novo* FA synthesis and elongase activities. Correlation matrix **(A)** of hepatic TAG levels and estimated activities of *de novo* synthesis (*De novo*) of FAs and elongases (elong) of SFAs, n-3, n-6, and n-7 PUFAs based on the abundance of FAs extracted from the livers of C57BL/6 mice given a high-carbohydrate control diet (HC) or a high-carbohydrate diet spiked with meldonium (HC + M), tetradecylthioacetic acid(HC + TTA), or meldonium + TTA (HC + M + TTA) for 21 days. The bar charts **(B)** illustrate the estimated delta 9 desaturase activities based on the abundance of FAs extracted from individual liver and pooled plasma samples and the hepatic FA synthesis activity. The bar charts **(C)** illustrate the delta estimated elongase activities of n-3, n-6, and n-7 PUFAs based on the abundance of FAs extracted from individual liver and pooled plasma samples. The bars in B, C, and D represent the mean of the individual values shown as dots. One-way ANOVA with Tukey's post hoc test was used to determine significant differences between the intervention groups. Different letters indicate statistical significance ($P < 0.05$) between groups. Abbreviations: FAs, fatty acids; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TAG, triacylglycerol.

4. Discussion

This study confirmed that TTA strongly attenuated meldonium-induced steatosis and demonstrated an association between liver TAG and changes in FA metabolism. TTA mediates its action at least in part by targeting the mitochondria and inducing mitochondrial biogenesis and β-oxidation.[46] The findings herein suggest that TTA also acts by other mechanisms, as TTA can prevent the accumulation of liver TAG under L-carnitine depletion, a condition where mitochondrial β-oxidation is inhibited. Our study demonstrates

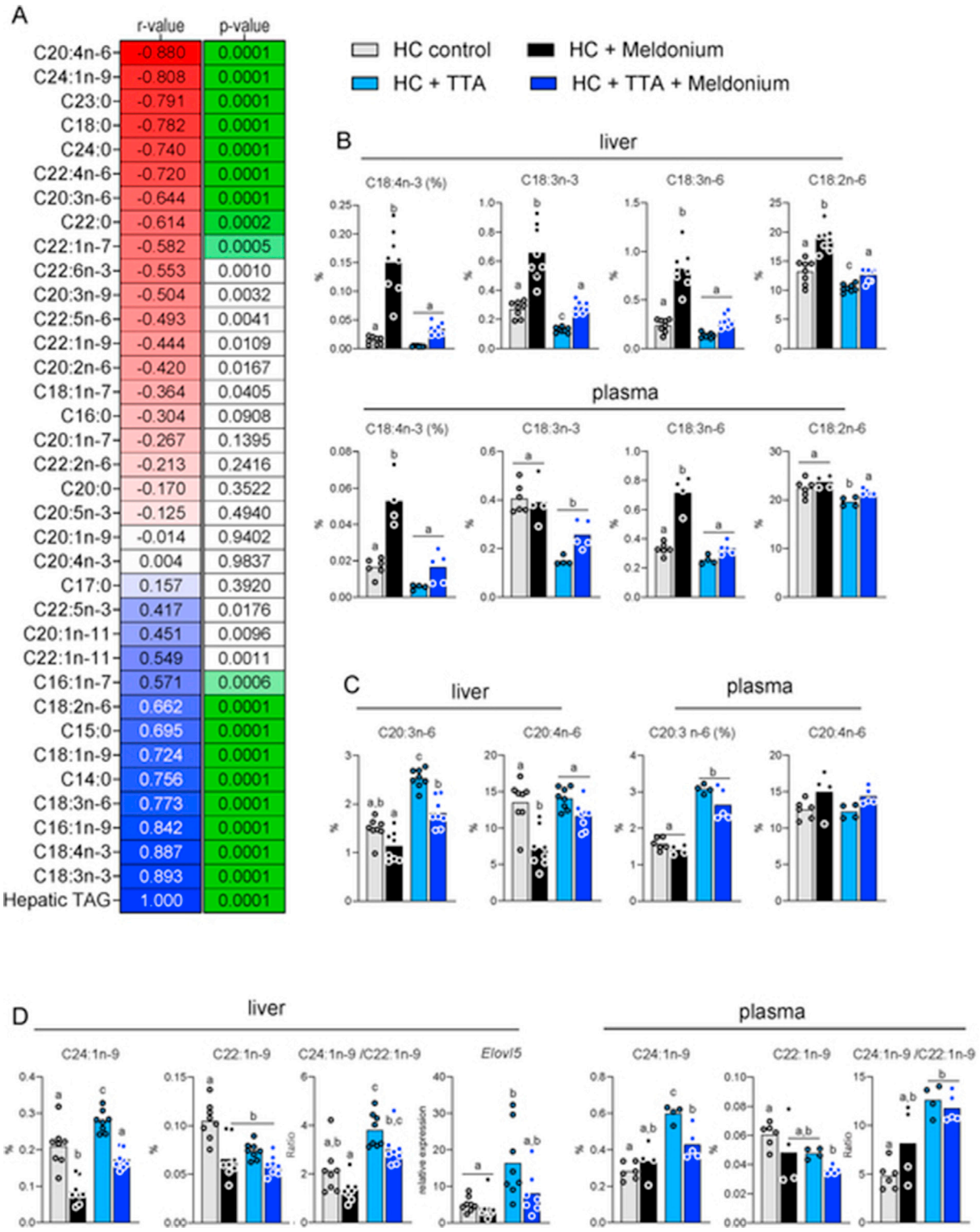

Fig. 6. Correlation between hepatic triacylglycerol levels and fatty acid indices. Hepatic triacylglycerol (TAG) levels were correlated with the relative abundance of hepatic fatty acids in C57BL/6 mice given a high-carbohydrate control diet (HC control) or a high-carbohydrate diet spiked with meldonium (HC + meldonium), tetradecylthioacetic acid (HC+TTA), or TTA+ meldonium (HC+TTA+ meldonium) for 21 days **(A–B)**. The bar charts **(C–D)** illustrate the relative abundance of fatty acids extracted from individual liver and pooled plasma samples and the hepatic relative expression of fatty acid elongase 5 (Elovl5). The bars in B, C, and D represent the mean of the individual values shown as dots. One-way ANOVA with Tukey's post hoc test was used to determine significant differences between the intervention groups. Different letters indicate statistical significance ($P<0.05$) among groups.

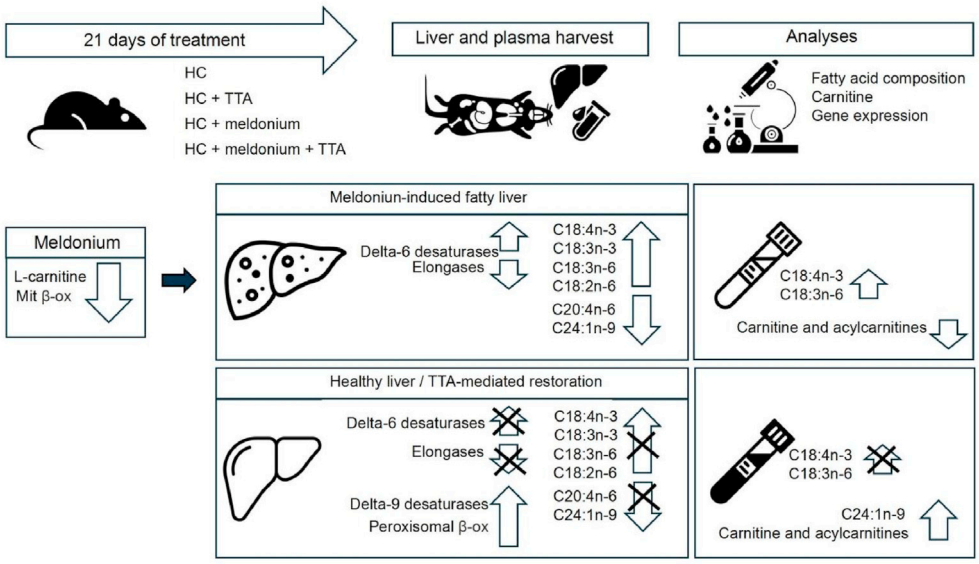

Fig. 7. The experimental model and summary of results. C57BL/6 mice were fed high-carbohydrate diets supplemented with meldonium, TTA, or a combination of both for 21 days. The study found that the meldonium-induced increase in hepatic triacylglycerol levels was accompanied by estimated activities of delta-6 desaturase and decreased estimated activities of elongases. TTA attenuated meldonium-induced triacylglycerol levels by 80% and restored the estimated delta-6 desaturase activities, and elongation of n-6 polyunsaturated FAs. Additionally, TTA treatment promoted peroxisomal β-oxidation and increased the estimated activities of delta-9 desaturase. TTA also mitigated the reduction in circulating carnitine and acylcarnitines as well as C24:1n-9 (nervonic acid), which was caused by meldonium. Icon used in this figure is downloaded from the Noun Project: Free Icons & Stock Photos for Everything (thenounproject.com). Abbreviations: FA, fatty acid; β-ox, beta-oxidation; TTA, tetradecylthioacetic acid.

that TTA can restore meldonium-mediated changes in D6D and elongation of FAs.

TTA is shown to reduce both plasma and liver TAG by increasing mitochondrial β-oxidation.[46] In line with the earlier finding that TTA-mediated stimulation of mitochondrial β-oxidation is associated with increased plasma acylcarnitine levels,[33] we observed higher levels of palmitoylcarnitine in the plasma from mice treated with TTA alone. However, TTA was not able to rescue the meldonium-mediated decrease in plasma palmitoylcarnitine. Meldonium is reported to inhibit L-carnitine biosynthesis, and hence carnitine levels and mitochondrial FA β-oxidation.[14,15] In line with this, we observed reduced plasma levels of γ-butyrobetaine, L-carnitine, and acylcarnitine in meldonium-treated mice, suggesting that meldonium treatment prevents the entry of FAs into the mitochondria. The hepatic capacity to oxidize palmitoyl-CoA to acid-soluble products in the presence of endogenously added carnitine as well as CPT2 capacity in liver homogenate were, however, not reduced by meldonium. TTA is known to increase hepatic mitochondrial β-oxidation and CPT2 activity, and *ex vivo* measured capacities of CPT2 and palmitoyl-CoA oxidation in the presence of L-carnitine were higher in mice treated with TTA and meldonium. However, there is no indication that TTA can restore the meldonium-mediated inhibition of L-carnitine synthesis as TTA did not rescue the meldonium-mediated decrease in plasma levels of γ-butyrobetaine and L-carnitine. Therefore, despite the increased capacity for mitochondrial β-oxidation, the FAs may be unable to enter the mitochondria *in vivo* in mice treated with meldonium and TTA. We found that both the capacity and gene expression of ACOX, the rate-limiting enzyme of peroxisomal β-oxidation, which unlike the mitochondrial β-oxidation, does not require L-carnitine, were increased by TTA in mice treated both in the absence and in combination with meldonium. TTA-induced peroxisomal β-oxidation can play a role when mitochondrial β-oxidation is reduced by L-carnitine depletion, but mitochondrial β-oxidation will quantitatively dominate over peroxisomal β-oxidation in liver cells under normal circumstances.

Increased lipogenesis and increased uptake of free FAs via the FA translocase (FAT/CD36) may contribute to the development of steatosis.[47] TTA was earlier shown to suppress hepatic lipogenesis in rats,[41] but neither FA synthesis activity in liver homogenate nor the lipogenesis index C16:0/C18:2n-6 were suppressed by TTA in the present study. Unexpectedly, both the lipogenesis index and FA synthesis activity were reduced in meldonium-treated mice and lipogenesis is most likely not involved in meldonium-induced steatosis nor the TTA-mediated effects.

In line with the reported increased D9D activity in patients with steatosis,[19,25] we here demonstrate that estimated liver activities of both SCD-16 (C16:1n-7/C16:0) and SCD-18 (C18:1n-9/C18:0) correlated with liver TAG. Earlier findings that mice with a targeted disruption in the *Scd1* isoform are resistant to diet-induced steatosis,[48] and inhibition of *Scd1* ameliorates hepatic steatoses in mice suggest that *Scd1* may be a pharmacological target.[49,50] However, in line with earlier studies,[32] TTA stimulated *Scd1* expression, and the meldonium-induced induction of estimated D9D activity was not restored by TTA in the present study. Hence, it is not likely that TTA mediates its ability to attenuate meldonium-induced steatosis via D9D.

Both n-6 and n-3 PUFA metabolism are controlled by D5D and D6D. In line with the reported higher estimated activities of D6D in individuals with obesity and MASH when compared to individuals with normal liver,[19] hepatic TAG levels correlated positively with estimated D6D activity in our study. Despite our finding that TTA induces the expression of *Fads2*, the meldonium-mediated increase in the hepatic D6D index was attenuated by the inclusion of TTA. The finding that estimated n-3 and n-6 D6D activities correlated with each other, as well as with the relative amounts of C18n-3 and C18n-6 FAs, suggest a coordinated regulation and corroborates this notion that estimated D6D activities may be markers of steatosis.

In line with the earlier reported negative association between the elongation of FAs and steatosis score in humans,[25] meldonium treatment led to reduced elongation indexes of both SFA as well as PUFAs. TTA was not able to restore the meldonium-mediated

reduction of the SFA elongation index but fully restored the elongation of n-6 and n-7 PUFAs. Furthermore, the n-3, n-6, and n-7 elongation indexes correlated positively with each other and negatively with hepatic TAG levels, suggesting a coordinated regulation of elongation of PUFAs and corroborating the notion that reduced elongation of PUFAs may be an indicator of hepatic steatosis.

The observed meldonium-induced changes in desaturation and elongation suggest an increased metabolism of C18:2n-6 (linoleic acid). Linoleic acid may be desaturated by D6D to γ-linolenic acid (C18:3n-6) that is elongated to dihomo-γ-linolenic acid (20:3n-6) and further desaturated to arachidonic acid (C20:4n-6) by D5D. Of note, hepatic TAG correlated positively with estimated n-6 D6D activity and the relative proportion of γ-linolenic acid, and negatively with estimated n-5 D6D activity as well as dihomo-γ-linolenic acid and arachidonic acid. This suggests that meldonium stimulates the conversion of dietary linolenic acid to γ-linolenic acid, whereas further metabolism into arachidonic acid is inhibited. The latter is in line with Yamada *et al.*[25] who reported decreased levels of dihomo-γ-linolenic acid and arachidonic acid in MASH patients. We, therefore, suggest that the development of steatosis is associated with increased D6D of linoleic acid combined with reduced elongation of n-6 PUFAs. The finding that the estimated hepatic D6D and n-6 elongation index, as well as that relative proportion of γ-linolenic acid in plasma mirrors the liver suggest that these may be considered as prognostic markers of hepatic TAG accumulation.

Dietary alpha-linolenic acid (C18:3n-3) is converted to eicosapentaenoic acid (C20:5n-3) in a similar manner via D6D to stearidonic acid (C18:4n-3) and via elongation to eicosatetraenoic acid (C20:4n-3) and subsequent D5 desaturation. Hepatic TAG correlated positively with n-3 D6D and stearidonic acid, but the negative correlation with n-3 elongation was rather weak ($r = -0.483$).

We observed a strong negative correlation between hepatic TAG and the relative proportion of nervonic acid (C24:1n-9). To our knowledge, nervonic acid has not before been associated with steatosis, but a negative association between plasma levels of nervonic acid and obesity is reported.[51] It is also demonstrated that supplementing a high-fat diet with nervonic acid attenuates obesity development and improves several metabolic parameters in mice.[52] Similar to an earlier report with TTA,[33] nervonic acid increases the levels of acylcarnitines and improves biomarkers of energy metabolism in the liver, including increased peroxisome proliferator-activated receptor α activation and FA oxidation.[52]

This study has some limitations. Firstly, the FA indices were based on FA composition measured in total fat. The relative proportion of hepatic phospholipids versus TAG will influence total FA composition in the liver, particularly when steatosis is developing. Still, our results are comparable with a human study stating that although the FA content of liver tissue was expected to increase in patients with advanced hepatic steatosis,[25] significant changes in the FA composition ratios suggested that not all FAs homogenously increase and concluded that FA components change depending on pathological differences in liver tissue in MAFLD patients. However, different turnover and compositions of different lipid fractions exist, and enzyme activities estimated by FA indices may not correlated with gene expression. For instance, SCD1 expression in human liver is positively correlated with a16:1n-7/16:0 ratio in liver phospholipids, triglycerides, cholesteryl esters, free FAs, and total lipids, whereas 18:1n-9/18:0 ratio was not correlated to liver SCD1 expression for any of these fractions.[53] Even though phospholipids are a preferable source in human blood, these are also a mixture of many sub-fractions with quite different FA compositions. Thus, cholesteryl esters are often used instead of phospholipids, especially in nutrition studies.[54] The low level of 18:3n-6 in phospholipids is often omitted from detection, and thus cholesteryl esters and triglycerides are used for the calculation of the omega-6 FA index.[19,54] In this study, 55%–85% of liver FAs are bound in TAGs, with the distribution of 10–18 carbon FAs in the range of 70%–90% in TAGs (except C18:0). Hence, the D6D index of total lipids is marginally affected by phospholipids or cholesteryl esters. On the other hand, C20:3n-6 and C20:4n-6 and longer PUFAs are predominantly bound in liver phospholipids and thus, D5D mainly corresponds to FA composition in this fraction. More detailed analyses of FAs of individual lipid fractions are needed to support these preliminary findings. Secondly, meldonium-induced steatosis does not reflect the complex pathogenesis of MAFLD. However, as mitochondrial dysfunction is observed in patients with fatty liver disease, and it is reported that mitochondrial dysfunction is linked to reduced mitochondrial FA β-oxidation in MALFD patients,[8,9] we think that meldonium-induced MAFLD may represent an interesting model.

5. Conclusions

In conclusion, TTA can attenuate meldonium-induced TAG levels by 80% and restore the meldonium-induced changes in estimated D6D and elongase activities. Circulating levels of γ-linolenic acid and estimated D6D and n-6 elongase indexes in plasma may be considered as prognostic markers for the development of fatty liver.

Data availability statement

Data are available by request to the corresponding authors.

Authors' contributions

Lise Madsen and **Rolf K Berge** contributed equally to this work and share senior authorship. **Bodil Bjørndal:** Writing – original draft, Formal analysis, Conceptualization. **Siri Lunde Tungland:** Writing – review & editing, Formal analysis. **Pavol Bohov:** Writing – review & editing, Formal analysis. **Magne O. Sydnes:** Writing – review & editing, Supervision. **Simon N. Dankel:** Writing – review & editing, Formal analysis. **Lise Madsen:** Writing – original draft, Supervision, Formal analysis, Conceptualization. **Rolf K Berge:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgements

The animal work was performed at The laboratory Animal Facility, a code facility at University of Bergen.

References

1. Pierantonelli I, Svegliati-Baroni G. Nonalcoholic fatty liver disease: basic pathogenetic mechanisms in the progression from NAFLD to NASH. *Transplantation*. 2019;103:e1–e13. https://doi.org/10.1097/TP.0000000000002480.
2. Younossi ZM, Golabi P, Paik JM, Henry A, Van Dongen C, Henry L. The global epidemiology of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH): a systematic review. *Hepatology*. 2023;77:1335–1347. https://doi.org/10.1097/HEP.0000000000000004.
3. Powell EE, Wong VW, Rinella M. Non-alcoholic fatty liver disease. *Lancet*. 2021;397:2212–2224. https://doi.org/10.1016/S0140-6736(20)32511-3.
4. Harrison SA, Bedossa P, Guy CD, et al. A phase 3, randomized, controlled trial of resmetirom in NASH with liver fibrosis. *N Engl J Med*. 2024;390:497–509. https://doi.org/10.1056/NEJMoa2309000.

5. Petta S, Targher G, Romeo S, et al. The first MASH drug therapy on the horizon: current perspectives of resmetirom. *Liver Int*. 2024;44:1526–1536. https://doi.org/10.1111/liv.15930.
6. Eslam M, Sanyal AJ, George J, International Consensus Panel. MAFLD: a consensus-driven proposed nomenclature for metabolic associated fatty liver disease. *Gastroenterology*. 2020;158:1999–2014(e1). https://doi.org/10.1053/j.gastro.2019.11.312.
7. Ramanathan R, Ali AH, Ibdah JA. Mitochondrial dysfunction plays central role in nonalcoholic fatty liver disease. *Int J Mol Sci*. 2022;23:7280. https://doi.org/10.3390/ijms23137280.
8. Gusdon AM, Song KX, Qu S. Nonalcoholic fatty liver disease: pathogenesis and therapeutics from a mitochondria-centric perspective. *Oxid Med Cell Longev*. 2014;2014:637027. https://doi.org/10.1155/2014/637027.
9. Moore MP, Cunningham RP, Meers GM, et al. Compromised hepatic mitochondrial fatty acid oxidation and reduced markers of mitochondrial turnover in human NAFLD. *Hepatology*. 2022;76:1452–1465. https://doi.org/10.1002/hep.32324.
10. Chen L, Li Y, Sottas C, et al. Loss of mitochondrial ATPase ATAD3A contributes to nonalcoholic fatty liver disease through accumulation of lipids and damaged mitochondria. *J Biol Chem*. 2022;298:102008. https://doi.org/10.1016/j.jbc.2022.102008.
11. Ibdah JA, Perlegas P, Zhao Y, et al. Mice heterozygous for a defect in mitochondrial trifunctional protein develop hepatic steatosis and insulin resistance. *Gastroenterology*. 2005;128:1381–1390. https://doi.org/10.1053/j.gastro.2005.02.001.
12. Selen ES, Choi J, Wolfgang MJ. Discordant hepatic fatty acid oxidation and triglyceride hydrolysis leads to liver disease. *JCI Insight*. 2021;6:e135626. https://doi.org/10.1172/jci.insight.135626.
13. Li N, Zhao H. Role of Carnitine in non-alcoholic fatty liver disease and other related diseases: an update. *Front Med (Lausanne)*. 2021;8:689042. https://doi.org/10.3389/fmed.2021.689042.
14. Liepinsh E, Vilskersts R, Skapare E, et al. Mildronate decreases carnitine availability and up-regulates glucose uptake and related gene expression in the mouse heart. *Life Sci*. 2008;83:613–619. https://doi.org/10.1016/j.lfs.2008.08.008.
15. Klusa V, Beitnere U, Pupure J, et al. Mildronate and its neuroregulatory mechanisms: targeting the mitochondria, neuroinflammation, and protein expression. *Medicina (Kaunas)*. 2013;49:301–309.
16. Degrace P, Demizieux L, Du ZY, et al. Regulation of lipid flux between liver and adipose tissue during transient hepatic steatosis in carnitine-depleted rats. *J Biol Chem*. 2007;282:20816–20826. https://doi.org/10.1074/jbc.M611391200.
17. Du ZY, Ma T, Liaset B, et al. Dietary eicosapentaenoic acid supplementation accentuates hepatic triglyceride accumulation in mice with impaired fatty acid oxidation capacity. *Biochim Biophys Acta*. 2013;1831:291–299. https://doi.org/10.1016/j.bbalip.2012.10.002.
18. Hodson L, Gunn PJ. The regulation of hepatic fatty acid synthesis and partitioning: the effect of nutritional state. *Nat Rev Endocrinol*. 2019;15:689–700. https://doi.org/10.1038/s41574-019-0256-9.
19. Walle P, Takkunen M, Männistö V, et al. Fatty acid metabolism is altered in non-alcoholic steatohepatitis independent of obesity. *Metabolism*. 2016;65:655–666. https://doi.org/10.1016/j.metabol.2016.01.011.
20. Wree A, Schlattjan M, Bechmann LP, et al. Adipocyte cell size, free fatty acids and apolipoproteins are associated with non-alcoholic liver injury progression in severely obese patients. *Metabolism*. 2014;63:1542–1552. https://doi.org/10.1016/j.metabol.2014.09.001.
21. Zhang J, Zhao Y, Xu C, et al. Association between serum free fatty acid levels and nonalcoholic fatty liver disease: a cross-sectional study. *Sci Rep*. 2014;4:5832. https://doi.org/10.1038/srep05832.
22. Puri P, Wiest MM, Cheung O, et al. The plasma lipidomic signature of nonalcoholic steatohepatitis. *Hepatology*. 2009;50:1827–1838. https://doi.org/10.1002/hep.23229.
23. Tomita K, Teratani T, Yokoyama H, et al. Plasma free myristic acid proportion is a predictor of nonalcoholic steatohepatitis. *Dig Dis Sci*. 2011;56:3045–3052. https://doi.org/10.1007/s10620-011-1712-0.
24. Dankel SN, Bjørndal B, Lindquist C, et al. Hepatic energy metabolism underlying differential lipidomic responses to high-carbohydrate and high-fat diets in male Wistar rats. *J Nutr*. 2021;151:2610–2621. https://doi.org/10.1093/jn/nxab178.
25. Yamada K, Mizukoshi E, Sunagozaka H, et al. Characteristics of hepatic fatty acid compositions in patients with nonalcoholic steatohepatitis. *Liver Int*. 2015;35:582–590. https://doi.org/10.1111/liv.12685.
26. Yamada K, Mizukoshi E, Seike T, et al. Serum C16:1n7/C16:0 ratio as a diagnostic marker for non-alcoholic steatohepatitis. *J Gastroenterol Hepatol*. 2019;34:1829–1835. https://doi.org/10.1111/jgh.14654.
27. Yamada K, Mizukoshi E, Sunagozaka H, et al. Response to importance of confounding factors in assessing fatty acid compositions in patients with non-alcoholic steatohepatitis. *Liver Int*. 2015;35:1773. https://doi.org/10.1111/liv.12755.
28. Røst TH, Haugan Moi LL, Berge K, Staels B, Mellgren G, Berge RK. A pan-PPAR ligand induces hepatic fatty acid oxidation in PPARalpha-/- mice possibly through PGC-1 mediated PPARdelta coactivation. *Biochim Biophys Acta*. 2009;1791:1076–1083. https://doi.org/10.1016/j.bbalip.2009.06.005.
29. Asiedu DK, al-Shurbaji A, Rustan AC, Björkhem I, Berglund L, Berge RK. Hepatic fatty acid metabolism as a determinant of plasma and liver triacylglycerol levels. Studies on tetradecylthioacetic and tetradecylthiopropionic acids. *Eur J Biochem*. 1995;227:715–722. https://doi.org/10.1111/j.1432-1033.1995.tb20193.x.
30. Raspé E, Madsen L, Lefebvre AM, et al. Modulation of rat liver apolipoprotein gene expression and serum lipid levels by tetradecylthioacetic acid (TTA) via PPARalpha activation. *J Lipid Res*. 1999;40:2099–2110.
31. Asiedu DK, Frøyland L, Vaagenes H, Lie O, Demoz A, Berge RK. Long-term effect of tetradecylthioacetic acid: a study on plasma lipid profile and fatty acid composition and oxidation in different rat organs. *Biochim Biophys Acta*. 1996;1300:86–96. https://doi.org/10.1016/0005-2760(95)00235-9.
32. Madsen L, Frøyland L, Grav HJ, Berge RK. Up-regulated delta 9-desaturase gene expression by hypolipidemic peroxisome-proliferating fatty acids results in increased oleic acid content in liver and VLDL: accumulation of a delta 9-desaturated metabolite of tetradecylthioacetic acid. *J Lipid Res*. 1997;38:554–563.
33. Bjørndal B, Alterås EK, Lindquist C, Svardal A, Skorve J, Berge RK. Associations between fatty acid oxidation, hepatic mitochondrial function, and plasma acylcarnitine levels in mice. *Nutr Metab (Lond)*. 2018;15:10. https://doi.org/10.1186/s12986-018-0241-7.
34. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*. 1959;37:911–917. https://doi.org/10.1139/o59-099.
35. Strand E, Bjorndal B, Nygard O, et al. Long-term treatment with the pan-PPAR agonist tetradecylthioacetic acid or fish oil is associated with increased cardiac content of n-3 fatty acids in rat. *Lipids Health Dis*. 2012;11:82. https://doi.org/10.1186/1476-511X-11-82.
36. Garras A, Asiedu DK, Berge RK. Subcellular localisation and induction of NADH-sensitive acetyl-CoA hydrolase and propionyl-CoA hydrolase activities in rat liver under lipogenic conditions after treatment with sulfur-substituted fatty acids. *Biochim Biophys Acta*. 1995;1255:154–160. https://doi.org/10.1016/0005-2760(94)00236-r.
37. Frøyland L, Asiedu DK, Vaagenes H, et al. Tetradecylthioacetic acid incorporated into very low density lipoprotein: changes in the fatty acid composition and reduced plasma lipids in cholesterol-fed hamsters. *J Lipid Res*. 1995;36:2529–2540.
38. Small GM, Burdett K, Connock MJ. A sensitive spectrophotometric assay for peroxisomal acyl-CoA oxidase. *Biochem J*. 1985;227:205–210. https://doi.org/10.1042/bj2270205.
39. Bremer J. The effect of fasting on the activity of liver carnitine palmitoyltransferase and its inhibition by malonyl-CoA. *Biochim Biophys Acta*. 1981;665:628–631. https://doi.org/10.1016/0005-2760(81)90282-4.
40. Madsen L, Berge RK. 3-Thia fatty acid treatment, in contrast to eicosapentaenoic acid and starvation, induces gene expression of carnitine palmitoyltransferase-II in rat liver. *Lipids*. 1999;34:447–456. https://doi.org/10.1007/s11745-999-0384-6.
41. Skorve J, al-Shurbaji A, Asiedu D, Björkhem I, Berglund L, Berge RK. On the mechanism of the hypolipidemic effect of sulfur-substituted hexadecanedioic acid (3-thiadicarboxylic acid) in normolipidemic rats. *J Lipid Res*. 1993;34:1177–1185.
42. Vernez L, Wenk M, Krähenbühl S. Determination of carnitine and acylcarnitines in plasma by high-performance liquid chromatography/electrospray ionization ion trap tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 2004;18:1233–1238. https://doi.org/10.1002/rcm.1470.
43. Vigerust NF, Bohov P, Bjørndal B, et al. Free carnitine and acylcarnitines in obese patients with polycystic ovary syndrome and effects of pioglitazone treatment. *Fertil Steril*. 2012;98:1620–1626 (e1). https://doi.org/10.1016/j.fertnstert.2012.08.024.
44. Simkhovich BZ, Shutenko ZV, Meirena DV, et al. 3-(2,2,2-Trimethylhydrazinium) propionate (THP)–a novel gamma-butyrobetaine hydroxylase inhibitor with cardioprotective properties. *Biochem Pharmacol*. 1988;37:195–202. https://doi.org/10.1016/0006-2952(88)90717-4.
45. Tsoko M, Beauseigneur F, Gresti J, et al. Enhancement of activities relative to fatty acid oxidation in the liver of rats depleted of L-carnitine by D-carnitine and a gamma-butyrobetaine hydroxylase inhibitor. *Biochem Pharmacol*. 1995;49:1403–1410. https://doi.org/10.1016/0006-2952(95)00019-v.
46. Berge RK, Tronstad KJ, Berge K, et al. The metabolic syndrome and the hepatic fatty acid drainage hypothesis. *Biochimie*. 2005;87:15–20. https://doi.org/10.1016/j.biochi.2004.11.011.
47. Zeng H, Qin H, Liao M, et al. CD36 promotes de novo lipogenesis in hepatocytes through INSIG2-dependent SREBP1 processing. *Mol Metab*. 2022;57:101428. https://doi.org/10.1016/j.molmet.2021.101428.
48. Ntambi JM, Miyazaki M, Stoehr JP, et al. Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity. *Proc Natl Acad Sci U S A*. 2002;99:11482–11486. https://doi.org/10.1073/pnas.132384699.
49. Li ZZ, Berk M, McIntyre TM, Feldstein AE. Hepatic lipid partitioning and liver damage in nonalcoholic fatty liver disease: role of stearoyl-CoA desaturase. *J Biol Chem*. 2009;284:5637–5644. https://doi.org/10.1074/jbc.M807616200.
50. Zhou Y, Zhong L, Yu S, Shen W, Cai C, Yu H. Inhibition of stearoyl-coenzyme A desaturase 1 ameliorates hepatic steatosis by inducing AMPK-mediated lipophagy. *Aging (Albany NY)*. 2020;12:7350–7362. https://doi.org/10.18632/aging.103082.
51. Pickens CA, Sordillo LM, Comstock SS, et al. Plasma phospholipids, non-esterified plasma polyunsaturated fatty acids and oxylipids are associated with BMI. *Prostaglandins Leukot Essent Fatty Acids*. 2015;95:31–40. https://doi.org/10.1016/j.plefa.2014.12.001.

52. Keppley LJW, Walker SJ, Gademsey AN, et al. Nervonic acid limits weight gain in a mouse model of diet-induced obesity. *FASEB J*. 2020;34:15314–15326. https://doi.org/10.1096/fj.202000525R.
53. Peter A, Cegan A, Wagner S, et al. Hepatic lipid composition and stearoyl-coenzyme A desaturase 1 mRNA expression can be estimated from plasma VLDL fatty acid ratios. *Clin Chem*. 2009;55:2113–2120. https://doi.org/10.1373/clinchem.2009.127274.
54. Vessby B, Gustafsson IB, Tengblad S, Berglund L. Indices of fatty acid desaturase activity in healthy human subjects: effects of different types of dietary fat. *Br J Nutr*. 2013;110:871–879. https://doi.org/10.1017/S0007114512005934.