

HMGR Inhibition Impairs Mitochondrial Fatty Acid Oxidation in Clinical and Preclinical Models: Therapeutic Targeting of Long-Chain Acylcarnitines

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Abstract

Background

Inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) by statins effectively lowers cholesterol levels but has also been suggested to impair mitochondrial metabolism and contribute to statin-associated adverse effects. Debate persists as to whether statin-associated muscle symptoms (SAMS) result primarily from systemic inhibition of the mevalonate pathway or from direct impairment of mitochondrial function by statins. We previously showed that tamoxifen-inducible, whole-body *Hmgcr*-knockout (*Hmgcr*-KO) mice develop severe metabolic disturbances and lethal liver injury associated with long-chain acylcarnitine accumulation. Here, we investigated whether a similar metabolic phenotype occurs in statin-treated patients and whether pharmacological lowering of acylcarnitine levels can mitigate the toxicity associated with HMGCR deficiency.

Methods

Blood plasma and muscle samples were obtained from healthy controls and patients receiving statin therapy. Statin concentrations, acylcarnitine profiles, and mitochondrial respiratory function were analysed in the collected samples. A tamoxifen-inducible, whole-body *Hmgcr*-KO mouse model was used to assess the effects of pharmacological acylcarnitine lowering.

Results

In muscle biopsies from statin-treated patients, fatty acid oxidation-linked mitochondrial respiration was reduced, and medium- and long-chain acylcarnitine concentrations were increased, whereas these changes were not evident in plasma. *Hmgcr*-KO mice similarly exhibited impaired mitochondrial fatty acid β -oxidation and respiration, accompanied by marked hepatic accumulation of long-chain and 3-hydroxy-acylcarnitines. Importantly, treatment with the acylcarnitine-lowering agent methyl-GBB markedly improved survival, prevented hypoglycaemia, reduced liver injury, preserved mitochondrial respiratory function, and normalized hepatic long-chain acylcarnitine profiles. In contrast, mevalonate treatment did not rescue the phenotype and increased the levels of several acylcarnitine species.

Conclusions

These findings identify a shared fatty acid oxidation–acylcarnitine phenotype associated with pharmacological and genetic HMGCR inhibition and demonstrate that targeting acylcarnitine accumulation can substantially mitigate the severe metabolic consequences of HMGCR deficiency. The findings support long-chain acylcarnitine accumulation as a mechanistically relevant consequence of HMGCR inhibition and suggest that acylcarnitine metabolism may represent a potential therapeutic target for statin-associated adverse effects.

Trial registration information: ClinicalTrials.gov registration number NCT07511686.

Highlights

Statin use is associated with impaired muscle FAO and higher long-chain acylcarnitine levels

HMGCR deficiency causes marked hepatic long-chain acylcarnitine accumulation

Acylcarnitine lowering preserves mitochondrial function and improves *Hmgcr*-KO survival

Acylcarnitine accumulation is a potential therapeutic target for statin-associated adverse effects

1 Introduction

Cardiometabolic pathologies persist as a substantial global health challenge and represent the primary driver of mortality [1]. Severe metabolic disturbances, characterized by the accumulation of cholesterol and lipid species, facilitate atherosclerotic plaque progression and subsequent cardiovascular disease. The most frequently prescribed metabolic drug class for hypercholesterolemia is statins, which pharmacologically target 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR; EC:1.1.1.34), the rate-limiting enzyme in the mevalonate pathway and cholesterol synthesis [2]. Although HMGCR inhibition effectively decreases circulating cholesterol levels and cardiovascular risk [3], increasing evidence indicates that impaired HMGCR activity also profoundly affects mitochondrial energy metabolism and may contribute to adverse reactions such as myopathy, which represents one of the most common reasons for treatment discontinuation [4], [5]. The specific biochemical and molecular pathways underlying these negative reactions are not yet fully understood, and questions about the causal relationships between statin therapy and undesirable effects have been raised [6], [7]. Debate persists regarding whether statin-associated muscle symptoms (SAMSs) emerge from a systemic reduction in lipid levels or a direct statin-induced impairment of mitochondrial function.

Currently, HMGCR is increasingly viewed as a metabolic regulator rather than only an enzyme that regulates cholesterol levels. The pleiotropic consequences of HMGCR inhibition include increased risks of T2D, elevated HbA1c levels, and greater adiposity [8]. Tissue-specific and inducible *Hmgcr* knockout (KO) mouse models in liver, muscle, and adipose tissues have demonstrated that a disruption of the mevalonate pathway results in severe metabolic disturbances, including impaired fatty acid β -oxidation, hypoglycaemia, mitochondrial dysfunction, rhabdomyolysis, lipodystrophy, and organ failure [9], [10], [11], [12]. Recently, we conducted multi-omics analyses of tamoxifen-inducible whole-body *Hmgcr*-KO mice [13] and observed that the loss of HMGCR activity rapidly induces lethal liver injury. The detrimental mechanisms leading to this outcome include a rapid disruption of mitochondrial fatty acid β -oxidation and defective peroxisomal fatty acid oxidation, which result in the accumulation of large amounts of long-chain acylcarnitines, toxic lipid intermediates known to impair mitochondrial respiration and ATP production [14]. Characterizing the effects of genetic and pharmacological HMGCR inhibition may help identify new biochemical markers and therapeutically relevant pathways.

We hypothesized that long-chain acylcarnitine accumulation could be responsible for the disruption of mitochondrial and peroxisomal metabolism and subsequent liver damage in animals. The existing preclinical evidence indicates that pharmacological modulation of acylcarnitine pathways might mitigate mitochondrial injury and restore the cellular energy balance [14]. Consequently, reducing long-chain acylcarnitine concentrations could be a therapeutic approach to counteract the lethal phenotype observed with HMGCR deficiency and potentially address statin-associated adverse effects. Therefore, in the present investigation, we characterized the perturbations in acylcarnitine metabolism in tamoxifen-inducible, whole-body *Hmgcr*-KO mice [13]. We evaluated whether the life-threatening accumulation of long-chain species could be prevented by mevalonate [9], [11] or the experimental acylcarnitine-lowering agent 4-[ethyl(dimethyl)ammonio]butanoate (methyl-GBB) [15], [13]. Furthermore, we collected human muscle biopsy samples from patients on statin therapy to determine drug levels, evaluate the mitochondrial respiratory capacity, and map the tissue acylcarnitine landscape.

2 Methods

2.1 Ethics statements

Clinical samples were collected in accordance with the principles of the Declaration of Helsinki and approved by the Ethics Committee for Clinical Research at the Development Society of Pauls Stradiņš Clinical University Hospital (Approval Number 270223-30L). This trial is registered at ClinicalTrials.gov under registration number NCT07511686. The ClinicalTrials.gov registration was completed retrospectively. All participants provided written informed consent before inclusion.

All experimental procedures involving animals were performed in accordance with the guidelines reported in the EU Directive 2010/63/EU and local laws and policies. All procedures were approved by the Latvian Animal Protection Ethical Committee of the Food and Veterinary Service in Riga, Latvia. All studies involving animals are reported in accordance with the ARRIVE guidelines [16].

2.2 Clinical study

A single-centre observational case–control study was conducted at the Latvian Cardiology Center of Pauls Stradiņš Clinical University Hospital in hospitalized patients, outpatients, and healthy controls. Adult participants (≥ 18 years) were Caucasian and were recruited from August 2023 to October 2024. The study population was divided into three groups: Group A, patients with elevated creatine kinase (CK) levels $> 1.5\times$ above the upper limit of normal (women > 325 U/L and men > 504 U/L) with or without muscle symptoms that have no other obvious explanation (including intense physical activity in the past 7 days); Group B, statin users—patients receiving stable high-intensity statin therapy (40–80 mg atorvastatin or 20–40 mg rosuvastatin) for at least 8 weeks without any muscle symptoms; and Group C, the Control group—individuals who had not used any lipid-lowering therapy within the past year and had no unexplained muscle symptoms. The exclusion criteria included individuals with diseases or conditions affecting muscle metabolism or CK levels, recent intense physical activity (during the past 7 days), acute cardiovascular events, severe systemic illness, excessive alcohol use, the use of

medications or substances known to cause myotoxicity, and the use of the carnitine uptake inhibitor meldonium [17] within the past 2 months. A full list of inclusion and exclusion criteria, including group-specific lipid-lowering medications, is provided in the Supplement: Inclusion and exclusion criteria for clinical study groups.

Baseline data were collected during a single study visit. The information recorded included demographic characteristics (age and sex), clinical variables (comorbidities and medication/supplement use characteristics), and lifestyle factors (alcohol use and the presence of intensive physical activity). All the variables were defined prior to analysis. Continuous variables included age, laboratory parameters, and treatment-related variables. Categorical variables included sex, comorbidities, medication use, and lifestyle factors. Venous blood samples were collected from all participants for long-term plasma storage at ultralow temperatures. Specifically, 7 mL of blood was collected into a tube containing EDTA and immediately centrifuged, and the plasma was aliquoted into at least three tubes and stored at -80°C . These samples were used for the acylcarnitine analysis, while a portion of the aliquots was retained for long-term storage for future research. A second 7 mL blood sample was collected into a tube and used for routine biochemical analyses performed in the accredited laboratory of Pauls Stradins Clinical University Hospital (PSCUH). Clinical chemistry assays included measurements of total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, CK, alanine aminotransferase (ALT), and creatinine levels. The levels of non-esterified fatty acids (NEFAs) were measured using a commercially available kit from Fujifilm Wako Chemicals Europe GmbH (Neuss, Germany). Where applicable, previously recorded laboratory values were also obtained from the medical records. Predefined inclusion and exclusion criteria were strictly applied to minimize bias.

Participants were offered the opportunity to undergo a skeletal muscle biopsy and provided separate written informed consent for this procedure. Muscle tissue samples were obtained under local anaesthesia from the forearm muscle of the non-dominant arm. Muscle biopsies were performed at PSCUH using standard procedures. Immediately after collection, the samples were transported to the Latvian Institute of Organic Synthesis. During transportation, the muscle tissue was placed in a sealed container filled with buffer solution and kept on ice.

2.3 Experimental animals

Generation and genotyping of *Hmgcr* floxed and *Hmgcr* knockout mice

The *Hmgcr* floxed mouse line was generated by CRISPR/Cas9-mediated genome engineering, as previously described [13]. *Hmgcr* conditional knockout (KO, *Hmgcr*^{LoxP/LoxP}-*Rosa26*^{WT/Cre}) mice were obtained by crossing heterozygous double-mutant (*Hmgcr*^{WT/LoxP}-*Rosa26*^{WT/Cre}) mice with homozygous *Hmgcr* floxed mice (*Hmgcr*^{LoxP/LoxP}-*Rosa26*^{WT/WT}), followed by tamoxifen treatment to induce gene editing. The primers used for genotyping the *Hmgcr* and *Rosa26Cre* loci are listed in Supplementary Table 1. *Hmgcr* genotyping yielded specific amplicons corresponding to wild-type (157 bp), homozygous floxed (217 bp), or heterozygous (217 and 157 bp) alleles. *Rosa26Cre* genotyping identified a wild-type

allele (198 bp) and a mutant allele (150 bp). PCR products were resolved on 2% agarose gels at 90 V for 60 min.

Experimental animals

A total of 45 adult 12-week-old male mice were used in the experiment, including 11 floxed mice and 34 *Hmgcr* conditional KO mice (12 *Hmgcr*-KO mice in the Control group, 12 *Hmgcr*-KO mice in the methyl-GBB-treated group, and 10 *Hmgcr*-KO mice in the mevalonate-treated group). The animals were maintained under standard conditions (21–23°C, 12 h light/dark cycle) with ad libitum access to standard chow (V1554-000, Sniff Spezialdiäten, Germany) and water in individually ventilated cages (Allentown, USA). Cages contained wood chip bedding (EcoPure Shavings, Datesand, UK), nesting material, wooden blocks (TAPVEI, Estonia), and red polycarbonate shelters (Safe Harbor Mouse Retreat, Animalab, Poland) for environmental enrichment. Mice were group-housed (3–5 per cage), and floxed and *Hmgcr*-KO mice were housed separately.

Hmgcr-KO induction and treatment: Global *Hmgcr* deletion was induced by feeding mice a tamoxifen-supplemented diet (A155T70212, 210 mg/kg tamoxifen citrate; ssniff Spezialdiäten, Germany), as described previously [13]. The mice received methyl-GBB (5 mg/kg) in their drinking water starting 3 days before tamoxifen treatment and continuing until the end of the experiment to mitigate acute liver injury. Mevalonate (150 mg/kg) was used as a reference compound and was administered subcutaneously once daily from tamoxifen diet initiation until study termination.

2.4 Blood and organ collection

Following the commencement of tamoxifen treatment, blood samples (20 µl) were collected from the tail vein on Days 5, 7, 9, and 11 to monitor longitudinal alterations in plasma alanine aminotransferase (ALT) activity. At the end of the experimental period, the mice were humanely euthanized by decapitation to enable subsequent harvesting of tissues and organs, particularly when the plasma ALT activity exceeded 300 IU in *Hmgcr*-KO mice. The tissues were harvested between Days 5 and 12 following the initiation of the tamoxifen-supplemented diet. At the experimental endpoint, multiple specimens were obtained, including plasma for systematic biochemical profiling and liver tissue for biochemical assessments, mitochondrial respiratory analysis, gene expression mapping, and histological evaluations.

2.5 Quantification of statin levels in human plasma and muscle samples

Stock solutions of rosuvastatin and atorvastatin were prepared at appropriate concentrations and combined to obtain a mixed working solution. For the plasma analysis, calibration standards were prepared by spiking the mixed stock solution into blank human plasma, followed by serial dilution with plasma to produce final concentrations ranging from 0.2 to 50 nM for both analytes. Protein precipitation was used for the preparation of the calibration standards and plasma samples. The samples were diluted 10-fold with acetonitrile/methanol (3:1, v/v), vortexed, and centrifuged. The supernatant was collected and subjected to ultra-performance liquid chromatography–tandem mass spectrometry

(UPLC–MS/MS) analysis. For the muscle analysis, calibration standards were prepared separately in acetonitrile/methanol (3:1, v/v) over the concentration range of 0.3–4 nM for both analytes. Human muscle samples were homogenized in a buffered KH_2PO_4 solution (pH 4.9), followed by deproteinization with an acetonitrile/isopropanol/methanol mixture (3:1:1, v/v/v) and centrifugation (final tissue-to-solvent ratio of 1:30, w/v). The supernatants were analysed directly by UPLC–MS/MS without further dilution.

Chromatographic separation was performed using an ACQUITY UPLC H-Class system (Waters Corporation). An ACQUITY UPLC BEH C18 column was used for analyte separation. The column temperature was maintained at 30°C, and the flow rate was set to 0.4 mL/min. The mobile phase consisted of (A) 0.1% formic acid in water and (B) acetonitrile. The gradient elution program was as follows: 0–0.5 min, 95% A; 0.5–2.5 min, linear decrease to 2% A; 2.5–3.5 min, a hold at 2% A; 3.5–3.7 min, return to 95% A; and equilibration at 95% A until 5.0 min. Mass spectrometric detection was performed using a triple quadrupole mass spectrometer (Xevo TQ-S micro, Waters Corporation) equipped with an electrospray ionization (ESI) source operated in positive ionization mode. The capillary voltage was set to 3.0 kV. The source temperature was 140°C, and the desolvation temperature was 600°C. Nitrogen was used as the desolvation gas at a flow rate of 800 L/h and as the cone gas at 50 L/h. MS/MS detection was performed in multiple reaction monitoring (MRM) mode. For rosuvastatin, the precursor ion at m/z 482 was monitored, with product ions detected at m/z 258 and 300. The cone voltage was set to 30 V, and the collision energy was 33 eV for the quantifier transition. Mass spectrometric monitoring of atorvastatin was achieved by tracking the m/z 559 precursor ion and its corresponding m/z 440 fragment. Quantification was performed using a 30 V cone voltage and a 22 eV collision energy for the specific transition.

2.6 RT–qPCR

Total RNA was extracted from liver tissues using TRI reagent (Sigma–Aldrich, St. Louis, MO, USA), and a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) was used to synthesize the first-strand cDNA. The qPCR analysis of gene expression was performed by adding SYBR® Green Master Mix (Applied Biosystems™) to each sample together with forwards and reverse primers specific for the HMG-CoA reductase (*Hmgcr*) N- and C-termini; acyl-CoA oxidase 1 (*Acox1*); carnitine palmitoyltransferase 1 (*Cpt1*); fatty acid-binding protein 1 (*Fabp1*); enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase (*Ehhadh*); acetyl-CoA acyltransferase 1B (*Acaa1b*); hydroxyacyl-coenzyme A dehydrogenase (*Hadh*); carnitine palmitoyltransferase 2 (*Cpt2*); hydroxysteroid 17-beta dehydrogenase 6 (*Hsd17b6*); and acyl-coenzyme A thioesterase 3 (*Acot3*). Reactions were performed using a magnetic induction cycler (MIC) (BioMolecular Systems, Australia). In accordance with the manufacturer's instructions, the reaction conditions were 50°C for 2 minutes and 95°C for 5 minutes, followed by 60 cycles of 95°C for 15 seconds and 60°C for 60 seconds, and the melting curve was analysed at the end of the cycles (72–95°C, 0.3°C/sec to ensure the specificity of the amplicon). The primer sequences are available in Supplementary Table 2.

2.7 Clinical chemistry assays

Biochemical parameters were analysed in mouse plasma and liver homogenates using commercially available assay kits according to the manufacturer's instructions. Kits for measuring cholesterol, triglycerides, glucose, and alanine transaminase (ALT) levels were obtained from the Instrumentation Laboratory (Milano, Italy). Lactate levels were determined using a kit from Roche Diagnostics (Mannheim, Germany). Catalase activity was assessed spectrophotometrically based on hydrogen peroxide decomposition, as previously described [18].

2.8 Determination of the acylcarnitine profile

The quantification of acylcarnitines in human plasma and muscle and mouse liver samples was performed using UPLC–MS/MS, as described previously [19], with some modifications. The method was further extended to include the quantification of the following long-chain 3-hydroxyacylcarnitines: 3-hydroxytetradecanoyl-L-carnitine (C14-OH), 3-hydroxyhexadecanoyl-L-carnitine (C16-OH), 3-hydroxyoctadecanoyl-L-carnitine (C18:0-OH), hydroxyoleoyl-L-carnitine (C18:1-OH), and hydroxylinoleylcarnitine (C18:2-OH). In the absence of authentic standards for C18:1-OH and C18:2-OH, their concentrations were quantified using the calibration curve generated for C18:0-OH. The calibration ranges, MRM transitions, and compound-specific mass spectrometric parameters are provided in Supplementary Table 3. A Waters Acquity UPLC H-Class chromatograph was coupled to a Waters Xevo TQ-S tandem mass spectrometer. Chromatographic separation was performed on a Waters Acquity UPLC BEH HILIC (2.1 × 100 mm, 1.7 µm) column using gradient elution. Solvent A consisted of 10 mM ammonium acetate with 0.2% formic acid in water, and solvent B was acetonitrile. The initial mobile phase composition was 10% solvent A, which was linearly increased to 20% solvent A in 6 min, followed by a linear increase to 65% at 7.3 min and a hold until 8.0 min. The mobile phase was then returned to the initial conditions at 8.3 min, and the column was re-equilibrated until 10.0 min. The flow rate was maintained at 0.5 mL/min throughout the analysis. The column temperature was maintained at 30°C. Data acquisition was performed in positive electrospray ionization (ESI+) mode and using multiple reaction monitoring (MRM). The ion source parameters were as follows: source temperature, 120°C; capillary voltage, 2.5 kV; desolvation gas temperature, 600°C; and desolvation and cone gas flow rates 800 L/h and 150 L/h, respectively. Each compound was individually infused to obtain specific MRM transitions and cone/collision voltages.

Calibration standards were prepared in acetonitrile/methanol (3:1, v/v). The muscle and liver samples were extracted by homogenization in a buffered KH₂PO₄ solution (pH 4.5), followed by deproteinization with an acetonitrile/isopropanol/methanol mixture (3:1:1, v/v/v) and centrifugation (final tissue-to-solvent ratio of 1:30, w/v). The supernatants were further diluted with acetonitrile/methanol (3:1, v/v) and subjected to UPLC–MS/MS. The plasma samples were deproteinized with an acetonitrile/methanol mixture (3:1, v/v) and diluted 200- or 400-fold prior to UPLC–MS/MS analysis.

2.9 Assessment of mitochondrial function

Mitochondrial respiration was measured at 37°C using Oxygraph-2k (O2k; Oroboros Instruments, Innsbruck, Austria) high-resolution respirometers with 2 mL chambers in MiR05 respiration medium [20]

supplemented with 20 mM creatine (MiR05Cr). Mitochondrial function was determined in saponin-permeabilized ($50 \mu\text{g}\cdot\text{mL}^{-1}$) human muscle fibres (1–2 mg wet weight in the O2k chamber) or mouse liver tissue homogenates, which were prepared using a Teflon-glass pestle at a concentration of $40 \text{ mg}\cdot\text{mL}^{-1}$, with the final concentration of tissue in the O2k chambers being $0.5 \text{ mg}\cdot\text{mL}^{-1}$. For patient muscle biopsy samples, a high-oxygen regimen ($\sim 420 \mu\text{M}$) was used to avoid oxygen limitation and to ensure that the maximum oxidative phosphorylation capacity was measured.

Adenosine diphosphate (ADP, 5 mM) and Complex I (CI) substrates (NADH (N)-pathway), malate (M; 0.5 mM), pyruvate (P; 5 mM), and glutamate (G; 10 mM) were added stepwise to study oxidative phosphorylation (OXPHOS)-dependent respiration. Afterwards, the Complex II (CII) substrate succinate (S; 10 mM) was added to reconstitute convergent CI&II-linked respiration (NS pathway). Carboxyatractyloside (CATR, 5 μM , an inhibitor of adenine nucleotide translocator) was added to assess respiration in the LEAK state. Finally, titration with the uncoupler (U) carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was performed in steps of 0.5 μM to assess the maximal capacity of electron transfer (ET).

In addition, fatty acid oxidation (FAO)-dependent mitochondrial respiration in the LEAK state was measured using palmitoylcarnitine (10 μM) and 0.5 mM malate as substrates (FADH₂(NADH) pathway; F(N) pathway). ADP (5 mM) was added to assess respiration via OXPHOS. Pyruvate and glutamate (5 mM and 10 mM, CI, N pathway) were then added to measure respiration associated with the FN pathway. Succinate (10 mM, S-pathway) was added to reconstitute convergent FNS-linked respiration. Afterwards, titration with the uncoupler CCCP was used to assess the maximal capacity of the ET system. Then, rotenone (0.5 μM , inhibitor of CI) and antimycin A (2.5 μM , inhibitor of CIII) were added to determine the CII-linked OXPHOS capacity and residual oxygen consumption (ROX), respectively. Complex IV (CIV)-linked respiration was measured by reoxygenating the medium and adding ascorbate (2 mM) and N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD, 0.5 mM). The chemical background oxygen consumption induced by the auto-oxidation of TMPD and ascorbate reactions was assessed after the inhibition of CIV by sodium azide (100 mM) and reoxygenation.

All reagents, substrates, and inhibitors except for ADP (Calbiochem, San Diego, CA, USA) were purchased from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO, USA).

2.10 Histology

Mouse liver samples were embedded in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands), frozen in a dry ice/ethanol slurry, and stored at -20°C . Cryosections (10 μm) were prepared using a Thermo Scientific™ HM525 NX Cryostat (Thermo Scientific, Waltham, MA, USA), mounted on Superfrost Plus microscope slides (Thermo Scientific, Waltham, MA, USA), and stored at -20°C until staining.

For haematoxylin and eosin (H&E) staining, the sections were fixed with 96% ethanol for 10 min, rinsed with tap water, and stained with Shandon™ Instant Haematoxylin (Thermo Scientific) for 2 min. After

being washed, the sections were differentiated in Nu-Clear I and blued using Bluing Reagent (Thermo Scientific). Following a brief rinse with 96% ethanol, the cytoplasm was counterstained with alcoholic Shandon™ Eosin-Y Stain (Thermo Scientific). The sections were dehydrated through graded ethanol solutions, cleared in xylene, and mounted with DPX mounting medium (Sigma–Aldrich, St. Louis, MO, USA). Brightfield images were acquired using a Leica DMI8 inverted light microscope equipped with a Leica HC PL APO 10×/0.45 objective (Leica Microsystems, Wetzlar, Germany).

2.11 Mouse phenotyping system

Three to five days before initiating tamoxifen treatment, the mice were individually housed in metabolic and behavioural phenotyping chambers (Promethion Core Caging System; Sable Systems™, Las Vegas, NV, USA) and maintained on a standard 12 h light/dark cycle until the humane endpoint (HEP) was reached. This system consisted of 16 metabolic chambers identical to the home cages, including bedding and enrichment chambers. The system measured food and water intake, animal activity (total locomotion and voluntary running), and gas exchange rates (CO_2 , O_2 , and energy expenditure). Cages were equipped with load cell-based balances for the ad libitum monitoring of food and water intake, infrared beam break sensors for quantifying spontaneous physical activity, and the Promethion CORE system for indirect calorimetry of air samples [21].

The raw data were collected for 7–20 days and processed using the Sable Systems™ Macro Interpreter (v23.6). Data assembly and statistical analyses were subsequently conducted in Python (JupyterLab environment) using custom-written scripts to process the annotated gas exchange, physical activity, and cumulative food and water intake datasets. The scripts and a detailed record of the package versions used in the analysis are available in a publicly accessible GitHub repository.

Carbohydrate and fatty acid oxidation rates were estimated from oxygen consumption (VO_2) and carbon dioxide production (VCO_2) using stoichiometric equations derived from indirect calorimetry, based on the non-protein respiratory quotient relationships described by Weir [22] and further refined by Livesey and Elia [23], where [carbohydrate oxidation = $4.585(\text{VCO}_2) - 3.226(\text{VO}_2)$] and [fatty acid oxidation = $1.695(\text{VO}_2) - 1.701(\text{VCO}_2)$].

2.12 Statistical analysis

Clinical data were processed using the open-source statistical software Jamovi (version 2.6). Continuous variables were assessed for normality using the Shapiro–Wilk test and a visual inspection of histograms and Q–Q plots. Normally distributed variables are presented as the means $\pm$ standard deviations (SDs), while non-normally distributed variables are presented as the medians and interquartile ranges (Q1–Q3). Comparisons between statin users and controls were performed using Welch’s independent-samples t test for normally distributed variables and the Mann–Whitney U test for non-normally distributed variables. Categorical variables are presented as counts and percentages. All the statistical tests were two-sided, and a p value < 0.05 was considered to indicate statistical significance. Analyses were performed using the available data without imputation.

For animal experiments, statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). The statistical significance of differences in survival curves was assessed using the log-rank (Mantel–Cox) test. Pairwise comparisons between the Flox and *Hmgcr*-KO mice in the Control groups were performed using the non-parametric Mann–Whitney U test. Differences among the *Hmgcr*-KO mice and treatment groups were evaluated using one-way ANOVA followed by Tukey’s post hoc test. P values less than 0.05 were considered significant. The data are shown as the means $\pm$ SEMs.

3 Results

3.1 Characteristics of participants in the clinical study

The study population comprised thirty-eight enrolled individuals. In accordance with the established study design and predefined inclusion criteria, participants were assigned to Group A (n = 6), Group B (n = 18), or Group C (n = 14). Given the small sample size of Group A and the absence of significant differences compared with Group B, these two groups were combined for subsequent analyses. Consequently, the analyses were performed using two groups: statin users (n = 24) and the control group (n = 14). Among statin users, rosuvastatin monotherapy was the most frequently used treatment regimen (56.5%), followed by rosuvastatin combined with ezetimibe (26.1%) and atorvastatin monotherapy (17.4%). The median duration of statin treatment was 12.0 months (Q1–Q3: 3.0–36.0 months). All participants were included in the primary analysis; however, the results of 4 participants were excluded from the final analysis for the reasons explained below (Fig. 1A). The occurrence of missing data across the study population was minimal. Notably, creatine kinase (CK) levels could not be obtained for 4 controls and 1 statin user, limiting the available CK dataset to 10 subjects in the control group and 22 individuals in the statin user cohort. In instances where data were missing, the corresponding sample sizes (n) have been explicitly specified in the table.

The baseline demographic and clinical characteristics of the study population are presented in Table 1. The median age of the participants was 57 (52–60) years in the control group and 61 (56–73) years in the statin user group, and the difference was not statistically significant. The proportion of male participants was similar between groups (62% of controls vs. 71% of statin users). Clinical characteristics, including low body weight, hypothyroidism, renal disease, liver disease, and vitamin D deficiency, were relatively uncommon and did not differ significantly between groups (data not shown). Muscle biopsy was performed in 62% of controls and 67% of statin users.

Statin users had significantly lower concentrations of total cholesterol and LDL cholesterol than controls. The median total cholesterol concentration was 3.5 mmol/L (3.1–4.1) in statin users compared with 5.3 mmol/L (4.8–5.8) in controls ($p < 0.001$). Similarly, LDL cholesterol concentrations were significantly lower in statin users ($p < 0.001$). No statistically significant differences were observed in HDL cholesterol, triglyceride, creatine kinase, creatinine, or ALT levels (Table 1).

Table 1
Baseline characteristics of the study participants

Patient characteristics	Control group (N = 13)	Statin users (N = 21)	p value
Age, years	57 (52–60)	61 (56–73)	0.085
Sex, n (%)			
Male	8 (62%)	15 (71%)	
Female	5 (38%)	6 (29%)	
Agreement to perform muscle biopsy, n (%)	8 (62%)	14 (67%)	
Plasma biochemistry			
Total cholesterol, mmol/L	5.3 (4.8–5.8)	3.5 (3.1–4.1)	< 0.001
LDL cholesterol, mmol/L	3.0 (2.5–3.4)	1.6 (1.3–1.8)	< 0.001
HDL cholesterol, mmol/l	1.7 (1.4–1.9)	1.2 (1.1–1.5)	0.089
Triglycerides, mmol/l	1.2 (1.0–1.4)	1.3 (0.8–1.4)	0.780
NEFA, mmol/L	0.5 (0.3–0.8)	0.4 (0.3–0.8)	0.831
Creatine kinase (CK), U/L	108 (102–113), n = 9	124 (83–291), n = 20	0.409
Creatinine, μ mol/L	79 (72–81)	83 (72–94)	0.435
ALT, U/L	24 (21–40)	31 (27–45)	0.103

The values are presented as medians (IQRs) or n (%), as appropriate. P values were calculated using the Mann–Whitney U test. The sample size (n) is indicated for parameters where data are missing for some subjects.

3.2 Statin treatment affects the acylcarnitine profile and FAO-linked respiration in human samples

We measured the concentrations of statins in the plasma and muscle samples from all participants to confirm patient adherence to the prescribed treatment. If statin concentrations were below the limit of quantification in plasma (LOQ; 0.2 nM), patients were excluded from the analysis. In 3 patients, atorvastatin and rosuvastatin were not detected in the plasma, and in 2 patients, both statins were measured. In the control group, one volunteer was excluded because an atorvastatin concentration of 4.66 nM was measured. The measured median plasma concentrations of rosuvastatin (n = 17) and atorvastatin (n = 6) in the statin user cohort were 1.86 and 5.49 nM (Fig. 1A), respectively. Compared

with previously published pharmacokinetic data, these plasma concentrations correspond to approximately 16–24 h after the last intake of the respective statin [24], [25], [26]. The muscle concentrations of statin analytes were above the limit of detection for only 3 rosuvastatin users (median 16.57 ng/g tissue) and 1 atorvastatin-treated patient (16.44 ng/g tissue).

High-resolution respirometry conducted on specimens from participants who underwent successful muscle biopsy revealed significant perturbations in mitochondrial function. An evaluation of the fatty acid oxidation (FAO)-dependent pathway in the LEAK state (Fig. 1B) demonstrated that FAO-linked respiration was twofold lower in statin users than in control subjects. This impairment in mitochondrial respiratory capacity persisted in the OXPHOS state (Fig. 1C), where the median respiration rate reached only 8.1 pmol O₂/(s*mg) in the statin-treated cohort compared to 14.9 pmol O₂/(s*mg) in the control group.

Acylcarnitine levels measured in the fasted state are recognized as markers of impaired fatty acid oxidation. Therefore, in our study, muscle samples were collected from patients and control subjects after an overnight fast. The total muscle content of long-chain acylcarnitines differed substantially between control subjects and statin users (median of 6.9 vs. 12.5 nmol/g; Fig. 1D), which is consistent with the significantly lower rate of fatty acid oxidation in muscle observed in statin users (Fig. 1B, C). An analysis of individual long-chain acylcarnitines revealed higher levels of all measured species from C14 to C18:1 in statin users (Fig. 1G, H, I, J, K), with the most pronounced 3- to 4-fold differences observed in the C18:0 and C18:1 levels (Fig. 1I, J). Similarly, muscle levels of medium-chain acylcarnitines were higher in statin users, with C8:0, C10:0, and C12:0 acylcarnitines showing 5- to 8-fold higher concentrations than in muscles from control subjects (Fig. 1E). In contrast, the muscle content of short-chain acylcarnitines was similar between the groups (Fig. 1F).

We next compared the changes observed in muscle with the concentrations of the corresponding acylcarnitines in plasma (Fig. 1L, M, N). Only a subset of volunteers agreed to donate muscle samples; therefore, the number of plasma samples was significantly greater than the number of muscle samples. The plasma levels of long- and medium-chain acylcarnitines were similar between the two groups, whereas short-chain acylcarnitine concentrations were only 20% higher in statin users than in controls. These findings are consistent with those of previous reports indicating that plasma acylcarnitine concentrations do not reflect changes in their concentrations in muscle.

3.3 Methyl-GBB prevents lethality and restores metabolic homeostasis in *Hmgcr*-KO mice

This study confirmed that the deletion of the *Hmgcr* gene leads to acute liver failure and significant metabolic disruptions in *Hmgcr*-KO mice, resulting in a lethal phenotype manifesting on Days 5–12. Treatment with methyl-GBB resulted in a notable improvement in survival rates compared to the *Hmgcr*-KO mice receiving the control treatment (**Fig. 2A**). Additionally, increased plasma levels of the liver enzyme alanine transaminase (ALT) indicated acute liver damage, whereas methyl-GBB treatment

effectively reduced the increase in ALT levels (**Fig. 2B**). Because of liver failure, a reduction in glucose levels was observed in the KO mice; however, methyl-GBB treatment prevented hypoglycaemia (**Fig. 2C**). Mevalonate treatment did not significantly reduce mortality, the increase in the ALT levels, or hypoglycaemia.

Plasma levels of long-chain and corresponding 3-hydroxy acylcarnitines were significantly increased in *Hmgcr*-KO mice by 4- and 6.5-fold, respectively, compared with those in floxed control animals. Methyl-GBB treatment substantially reduced acylcarnitine concentrations to levels comparable to those in floxed control mice (**Fig. 2D, E**). In contrast, mevalonate administration significantly increased long-chain and 3-hydroxy acylcarnitine levels in plasma by 2- and 3.5-fold, respectively, compared with the levels in the *Hmgcr*-KO mice receiving the control treatment (**Fig. 2D, E**). The levels of medium- and short-chain acylcarnitines did not differ significantly between the floxed and KO mice in the control groups. Methyl-GBB treatment reduced and mevalonate supplementation increased medium- and short-chain acylcarnitine concentrations, which is consistent with the changes in the long-chain acylcarnitine levels (**Fig. 2F, G**). In the KO mice, the average lactate level was decreased by 1.6-fold compared with that in the floxed control mice but was not affected by treatment (**Fig. 2H**). Notably, plasma cholesterol concentrations were slightly elevated in the *Hmgcr*-KO mice from all groups, regardless of treatment (**Fig. 2I**), reflecting the dysregulation of cholesterol reuptake in the liver. The biochemical analysis of plasma triglyceride levels revealed no significant differences among the treatment groups (**Fig. 2J**).

Figure 2. While *Hmgcr* deletion leads to acute lethal liver failure and metabolic disruption, treatment with methyl-GBB can significantly improve survival in male *Hmgcr*-KO mice. (A) According to the Kaplan–Meier plot, a few days after the induction of *Hmgcr*-KO, the mice reached the HEP, and treatment with methyl-GBB significantly increased the survival rate. (B) A substantial increase in plasma ALT levels indicates acute liver failure. Methyl-GBB treatment significantly reduced the elevated ALT level in the KO animals, whereas mevalonate had no effect. (C) A significant decrease in glucose concentration was observed, whereas methyl-GBB treatment did not lead to any reduction in glucose levels in KO animals. (D) Long-chain acylcarnitine (AC) and (E) long-chain-3OH AC concentrations were substantially elevated in KO mice, and treatment with methyl-GBB reduced the blood LCAC levels. (F, G) Medium- and short-chain AC concentrations were not reliably increased in the KO mice. Biochemical measurements of plasma (H) lactate, (I) cholesterol, and (J) triglyceride levels. Plasma samples were collected from floxed, KO, and KO mice after treatment (methyl-GBB or mevalonate) at the endpoint (Days 7–12). The data are shown as the means $\pm$ SEMs ($n = 8–11$ mice per group). The statistical significance of differences in the survival curves (A) was assessed using the log-rank (Mantel–Cox) test. Pairwise comparisons between the floxed and *Hmgcr*-KO mice in the Control groups (B–J) were performed using the non-parametric Mann–Whitney U test. Differences among the *Hmgcr*-KO mice and treatment groups were evaluated using one-way ANOVA followed by Tukey’s post hoc test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$). *Abbreviations*: HEP, humane endpoint; ALT, alanine transaminase; AC, acylcarnitine; *Hmgcr*-KO, *Hmgcr* knockout; Flox, floxed; Cont, control; MG, methyl-GBB; Mev, mevalonate.

3.4 Methyl-GBB treatment mitigates hepatic damage and acylcarnitine accumulation in *Hmgcr*-KO mice

Macroscopic observation and histological analysis revealed extensive hepatic damage in *Hmgcr*-KO control mice compared with *Hmgcr*-Flox mice (Fig. 3A, B). While *Hmgcr*-Flox mice displayed a normal hepatic architecture and cytoplasmic staining, the *Hmgcr*-KO liver exhibited increased cytoplasmic paleness and reduced nuclear density and inflammatory cell infiltration, which may suggest the presence of steatosis and hepatocellular injury, possibly involving necrotic processes (Fig. 3A). Macroscopic observations of the liver revealed pale patches or a pale colour throughout the liver (Fig. 3B). Treatment with methyl-GBB, but not mevalonate, resulted in substantial improvements in liver histology and macroscopic observations, indicating that experimental methyl-GBB therapy effectively alleviated hepatic damage and preserved the tissue architecture in the *Hmgcr*-KO model mice.

In *Hmgcr*-KO mice, the average mRNA levels of carboxyl (C)-terminal and amino (N)-terminal *Hmgcr* fragments were significantly reduced by 2.5- and 3.5-fold, respectively, compared with those in floxed control animals (Fig. 3C, D). In both the methyl-GBB- and mevalonate-treated groups, the mRNA levels of the C-terminal and N-terminal *Hmgcr* fragments were reduced to levels similar to those in the *Hmgcr*-KO mice, indicating that mice receiving the therapeutic interventions expressed similar defective *Hmgcr* transcripts (Fig. 3C, D).

Increased levels of long-chain acylcarnitines indicate disturbed mitochondrial fatty acid oxidation. The analysis of the hepatic tissue revealed a significant 5-fold increase in long-chain acylcarnitine levels in *Hmgcr*-KO mice (Fig. 3E), and the levels of long-chain 3-hydroxy acylcarnitines were increased 2.5-fold in *Hmgcr*-KO mice compared with floxed control mice (Fig. 3F). Methyl-GBB treatment substantially reduced the levels of both long-chain acylcarnitines and corresponding 3-hydroxy acylcarnitines to concentrations comparable to those in the control animals, whereas mevalonate treatment failed to normalize acylcarnitine accumulation (Fig. 3E, F).

Minimal quantities of medium-chain acylcarnitines were present in the hepatic tissue, and no significant alterations were detected across the experimental groups (Fig. 3G). In contrast, short-chain acylcarnitine concentrations were substantially elevated in *Hmgcr*-KO mice compared with those in floxed animals (Fig. 3H). Methyl-GBB treatment significantly reduced short-chain acylcarnitine levels, whereas mevalonate supplementation substantially increased their hepatic accumulation (Fig. 3H).

Given that the majority of catalases are located in peroxisomes, we measured catalase activity as a measure of the peroxisome count. Catalase activity was significantly lower in the livers of *Hmgcr*-KO mice than in those of floxed control mice; this reduction was partially reversed by methyl-GBB treatment but remained low in the livers of mevalonate-treated animals (Fig. 3I). Hepatic triglyceride concentrations were markedly elevated in *Hmgcr*-KO mice. Neither methyl-GBB nor mevalonate treatment significantly altered the triglyceride levels (Fig. 3J).

We performed an RT-qPCR analysis of several highly differentially expressed genes in these mice to confirm our previous transcriptomic findings of the reduced expression of PPAR α target genes in *Hmgcr*-KO mice [13] and to assess the effects of treatment on gene expression (Fig. 3K). Significantly decreased expression of genes involved in mitochondrial and peroxisomal fatty acid oxidation, including *Cpt1*, *Cpt2*, *Ehhadh*, *Acot3*, and *Fabp1*, was detected in the liver of *Hmgcr*-KO mice compared with floxed control animals. Methyl-GBB treatment, but not mevalonate supplementation, partially reversed the changes in the expression of all analysed genes towards the levels observed in the floxed control mice. The expression of *Hsd17b6*, *Acaa1B* and *Acot3* was significantly restored, whereas the expression of the remaining genes showed a consistent but nonsignificant trend towards normalization (see **Supplementary Fig. 1** for the results of the statistical tests). Although *Acox1* expression was substantially but non-significantly reduced in *Hmgcr*-KO mice ($p = 0.06$), methyl-GBB treatment increased its expression compared with that in untreated KO animals, resulting in expression levels exceeding those in the floxed control mice.

3.5 Methyl-GBB preserves mitochondrial respiratory function and normalizes hepatic acylcarnitine metabolism in *Hmgcr*-KO mice

The high-resolution respirometry analysis revealed a significant reduction in mitochondrial respiration rates in *Hmgcr*-KO mice based on the levels of Complex I and II substrates in the OXPHOS state (Fig. 4A, B). Moreover, the maximal electron transfer (ET) capacity and Complex IV activity slightly decreased, but the effect was not statistically significant (Fig. 4C, E). We also detected a reduced FAO rate in the OXPHOS state in the hepatic mitochondria of *Hmgcr*-KO mice (Fig. 4D). In the livers of methyl-GBB-treated mice, the mitochondrial respiratory capacity was preserved and was similar to that in the livers of floxed control animals (Fig. 4A). Additionally, we found that mevalonate treatment did not improve the respiratory capacity, which decreased similarly to that in the KO mice (Fig. 4A–E). The results from complete substrate–uncoupler–inhibitor titration protocols used to assess mitochondrial function are available in the Supplementary Information (**Supplementary Fig. 2**).

In the livers of *Hmgcr*-KO mice, we detected the substantial accumulation of long-chain acylcarnitines, with marked increases in the levels of C14, C16, C18:0, and C18:1 acylcarnitines compared with those in floxed control mice (Fig. 4F–I). Notably, C18:2 acylcarnitine levels did not increase in the KO animals and constituted the smallest fraction of the measured C18 species (~ 50 nmol/g). (Fig. 4J). Additionally, the levels of long-chain 3-OH acylcarnitines were elevated in the KO mice, with a significant accumulation of C14-OH and C18:0-OH species (Fig. 4K, M). Methyl-GBB treatment effectively prevented the hepatic accumulation of long-chain acylcarnitines and corresponding 3-OH species (Fig. 4F–N). In contrast, compared with vehicle treatment, mevalonate supplementation resulted in divergent effects, significantly increasing the hepatic concentrations of C18:1, C14-OH, and C18:1-OH acylcarnitines in the KO animals (Fig. 4I, K, N). These differential responses suggest that methyl-GBB specifically counteracts

acylcarnitine accumulation and related changes in the mouse liver, whereas mevalonate treatment exacerbates hepatic acylcarnitine dysmetabolism in mice with *HMGCR* deficiency.

3.6 HMGCR deficiency disrupts whole-body energy metabolism and reduces locomotor activity

The mice were housed in SABLE metabolic phenotyping cages throughout the experiment to assess the whole-body metabolic and behavioural consequences of *Hmgcr* deficiency (Fig. 5A–E). In the final 24 hours before the humane endpoint (HEP), *Hmgcr*-KO mice exhibited significantly reduced energy expenditure (Fig. 5A) and carbohydrate oxidation rates (Fig. 5B) during the dark cycle (active phase), indicating impaired systemic energy metabolism and glucose utilization compared with the floxed control mice. In contrast, the whole-body fatty acid oxidation rate (Fig. 5D) did not differ significantly between the groups. Food intake (Fig. 5E) during the active phase remained unchanged across all the experimental groups, indicating that reduced caloric intake did not account for the observed metabolic alterations. Furthermore, *Hmgcr*-KO mice displayed significantly lower locomotor activity (Fig. 5C) during the active phase, consistent with the increasing severity of the *Hmgcr*-deficient phenotype. Neither methyl-GBB nor mevalonate treatment fully reversed the alterations in whole-body metabolism observed in *Hmgcr*-KO mice.

4 Discussion

In this study, we show for the first time that decreasing long-chain acylcarnitine accumulation attenuates *Hmgcr* deficiency-induced acute liver failure and markedly prolongs survival. While hepatic *Hmgcr* KO predictably resulted in a severe metabolic disruption, mitochondrial dysfunction, and lethality between Days 5 and 12 [13], methyl-GBB treatment effectively normalized plasma and hepatic long-chain and 3-hydroxy-acylcarnitine levels, prevented hypoglycaemia, reduced plasma ALT levels, preserved Complex I-driven mitochondrial respiration, and restored the hepatic histology. In contrast, mevalonate treatment did not significantly reduce mortality or improve the liver condition and actually exacerbated plasma and hepatic acylcarnitine accumulation. Overall, these results indicate that methyl-GBB treatment protected *Hmgcr*-KO mice primarily by reducing acylcarnitine accumulation and improving mitochondrial function, thus mitigating liver injury. Notably, the biochemical changes induced by both the methyl-GBB and mevalonate treatments were not reflected by any improvements in whole-body metabolic function or locomotor activity in this severe *Hmgcr*-KO mouse model.

Tissue-specific knockout models have established that *Hmgcr* deletion induces cell death and severe pathological phenotypes in multiple cell types, including hepatocytes, myocytes, adipocytes, pancreatic β -cells, and T cells [9], [11], [12]. Previously, we demonstrated that in *Hmgcr*-KO hepatocytes, impaired FAO and long-chain acylcarnitine accumulation trigger mitochondrial dysfunction, ER stress, and secondary cellular stress responses [13]. Furthermore, *Hmgcr* deletion significantly decreased the number of peroxisomes and peroxisomal FAO. As shown in the present study, similar metabolic perturbations, namely, suppressed FAO and muscle acylcarnitine accumulation, occur in patients

receiving statins. These parallel findings suggest that HMGCR inhibition accounts for statin-induced mitochondrial dysfunction and thus likely the adverse metabolic side effects in skeletal muscle.

The effects of statins on HMGCR and off-target effects on muscle and other tissues depend on the properties of individual statins [27]. Based on preclinical data from atorvastatin-treated obese minipigs, the highest intracellular level of statin is detected in the liver, while its content in muscle is considered significantly lower [28]. Bioavailability data concerning statin concentrations in human muscle remain very limited. An analysis of the atorvastatin metabolite pattern in skeletal muscle revealed cumulative concentrations of atorvastatin and its acid-form metabolites of approximately 4 ng/g and approximately 14 ng/g for the lactone form [29]. Another study that used a relatively high dose (80 mg/d) reported muscle atorvastatin concentrations ranging from 2 to 23 ng/g tissue and reduced mitochondrial respiration at 4 and 8 weeks following treatment initiation [30]. In the present study, treatment with rosuvastatin and atorvastatin resulted in muscle concentrations of approximately 16 ng/g muscle. These concentrations are expected to be sufficient to inhibit HMGCR activity. Intracellularly, HMGCR is located in peroxisomes [31], mitochondria and the endoplasmic reticulum [32]; consequently, statins must penetrate these cellular organelles to inhibit HMGCR. *In vitro* and *in vivo* analyses of statin metabolism have shown that β -oxidation is the prevailing metabolic pathway for atorvastatin [33]. Because β -oxidation occurs exclusively in peroxisomes and mitochondria, the extensive metabolism of atorvastatin confirms its entry into these organelles, where it can induce disruptions in fatty acid oxidation and the electron transport chain.

The available evidence indicates that statins can inhibit mitochondrial electron transport chain function. The initial studies identified Complex III as a principal site of inhibition; however, direct statin-mediated inhibition was observed only at high micromolar concentrations that are not achievable in patient tissues [34], [30]. More recent evidence suggests that atorvastatin can directly inhibit Complex IV at nanomolar concentrations [30]. In the present study, we did not detect lower Complex IV activity in KO mice but detected a significantly lower FAO rate in the livers of KO mice and in human muscle samples from statin users. In both samples, we observed a substantial increase in long-chain acylcarnitine content, which serves as a marker of a reduced FAO rate. The accumulation of long-chain hydroxy acylcarnitines also indicates decreased β -oxidation activity rather than direct effects on the mitochondrial respiratory chain. This effect was observed in both KO mice and statin users, providing evidence that it is caused by decreased HMGCR activity rather than an off-target effect of statins. This study confirms our previous findings of reduced expression of PPAR α target genes in *Hmgcr*-KO mice [13]. The partial restoration of the expression of this gene following methyl-GBB treatment suggests that lowering long-chain acylcarnitine levels may alleviate the suppression of PPAR α -dependent transcription in the HMGCR-deficient liver. Notably, methyl-GBB treatment markedly increased the expression of *Acox1*, a direct downstream target gene that is transcriptionally upregulated and controlled by PPAR α . In previous studies of *Hmgcr*-KO mice, mevalonate treatment restored serum creatinine levels in muscle-specific KO mice [11] and ALT levels in liver-specific KO mice *in vivo* [9]. In *in vitro* models, *Hmgcr* deletion or inhibition can be rescued by the addition of mevalonate or GGPP to *Hmgcr*-KO T cells [35] and lovastatin-treated 3T3-L1 adipocytes [12]. From a clinical perspective, mevalonate supplementation to rescue

statin-induced adverse reactions is not a viable option because it can restore cholesterol synthesis, thereby compromising clinical efficacy. We used mevalonate in our mouse experiments to distinguish the effects of a deficiency in mevalonate and its related metabolites from those of the enzyme deficiency itself. In our *Hmgcr*-KO model, mevalonate did not attenuate the severity of the *Hmgcr*-KO phenotype; moreover, the levels of certain parameters, such as hepatic medium- and long-chain 3-hydroxyacylcarnitines, were higher in *Hmgcr*-KO mice than in untreated KO mice. This finding highlights the importance of enzyme activity in preventing the accumulation of the HMGCR substrate, HMG-CoA. HMG-CoA is also a substrate for HMG-CoA lyase, a mitochondrial and peroxisomal enzyme that catalyses its breakdown into acetoacetate and acetyl-CoA. In humans, HMG-CoA lyase deficiency causes recurrent, life-threatening episodes of hypoglycaemia and acidosis [36]. Similar to *Hmgcr*-KO models, liver-specific *HMG-CoA lyase*-KO mice develop hypoglycaemia and experience 100% mortality within 30 days of KO induction [37].

Despite the availability of novel treatments for lowering cholesterol concentrations, the use of statins continues to increase, exposing a large number of patients to the risk of side effects. Novel treatments that would help to prevent adverse reactions are necessary to gain the maximum benefit from the cardiovascular risk reduction by statins and simultaneously avoid unwanted side effects. The findings of higher tissue levels of long-chain acylcarnitines in *Hmgcr*-KO mice and statin-treated patients prompted the idea to use acylcarnitine-lowering treatment to delay or prevent the development of detrimental changes in *Hmgcr*-KO mice. In previous studies, we demonstrated that treatment with the acylcarnitine-lowering compounds meldonium and methyl-GBB prevents long-chain acylcarnitine-induced damage in models of myocardial ischaemia [38], diabetes (insulin resistance) [39], and atherosclerosis [15]. A similar effect was observed in a whole-body trimethyllysine hydroxylase epsilon (TMLHE) knockout mouse model [40]. In this study, we showed that preventing the accumulation of long-chain acylcarnitines via a methyl-GBB pretreatment significantly attenuated the *Hmgcr*-KO phenotype. Notably, this intervention preserved mitochondrial function and prevented the loss of peroxisomes, resulting in reduced liver damage, alleviated hypoglycaemia, and markedly improved survival. Based on these results, statin-treated patients who experience adverse muscle effects could benefit from acylcarnitine-lowering therapy.

5 Conclusions

Our findings indicate that both statin-induced and genetic *HMGCR* inhibition cause metabolic disturbances, resulting in impaired mitochondrial respiration and the accumulation of long-chain acylcarnitines, highlighting the critical role of HMGCR in maintaining metabolic homeostasis. Importantly, lowering acylcarnitine levels with methyl-GBB markedly improved survival and liver function in the *Hmgcr*-KO mouse model, suggesting that acylcarnitine accumulation is a key mediator of *HMGCR* deficiency-induced toxicity and is a promising therapeutic target for mitigating the adverse effects associated with HMGCR inhibition.

Declarations

Author contributions: CRediT

Maija Dambrova: Writing – original draft, Project administration, Methodology, Funding acquisition, Conceptualization. **Līga Zvejniece:** Writing – original draft, Methodology, Investigation, Conceptualization, Formal analysis. **Melita Ozola:** Writing – review & editing, Investigation, Formal analysis. **Arturs Petersons:** Writing – review & editing, Methodology, Formal analysis. **Anna Miteniece:** Writing – review & editing, Investigation, Formal analysis. **Baiba Svalbe:** Writing – review & editing, Investigation, Formal analysis. **Helena Cirule:** Writing – review & editing, Investigation. **Stanislava Korzh:** Writing – review & editing, Investigation. **Baiba Gukalova:** Writing – review & editing, Investigation. **Kristaps Krims-Davis:** Writing – review & editing, Investigation. **Eva Bolsteina:** Writing – review & editing, Investigation. **Gustavs Latkovskis:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Ilze Konrade:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Helgi B Schiöth:** Writing – review & editing, Conceptualization, Methodology, Investigation, Formal analysis. **Edgars Liepinsh:** Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Ethics approval and consent to participate

Clinical samples were collected in accordance with the principles of the Declaration of Helsinki and approved by the Ethics Committee for Clinical Research at the Development Society of Pauls Stradins Clinical University Hospital (Approval Number 270223-30L). This trial is registered at ClinicalTrials.gov under registration number NCT07511686. The ClinicalTrials.gov registration was completed retrospectively. All participants provided written informed consent before inclusion.

All experimental procedures involving animals were performed in accordance with the guidelines reported in the EU Directive 2010/63/EU and local laws and policies. All procedures were approved by the Latvian Animal Protection Ethical Committee of the Food and Veterinary Service in Riga, Latvia. All studies involving animals are reported in accordance with the ARRIVE guidelines.

Consent for publication

All authors have reviewed the final version of the manuscript and approved it for publication.

Availability of data and material

Data availability

Data will be made available on reasonable request

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Figures

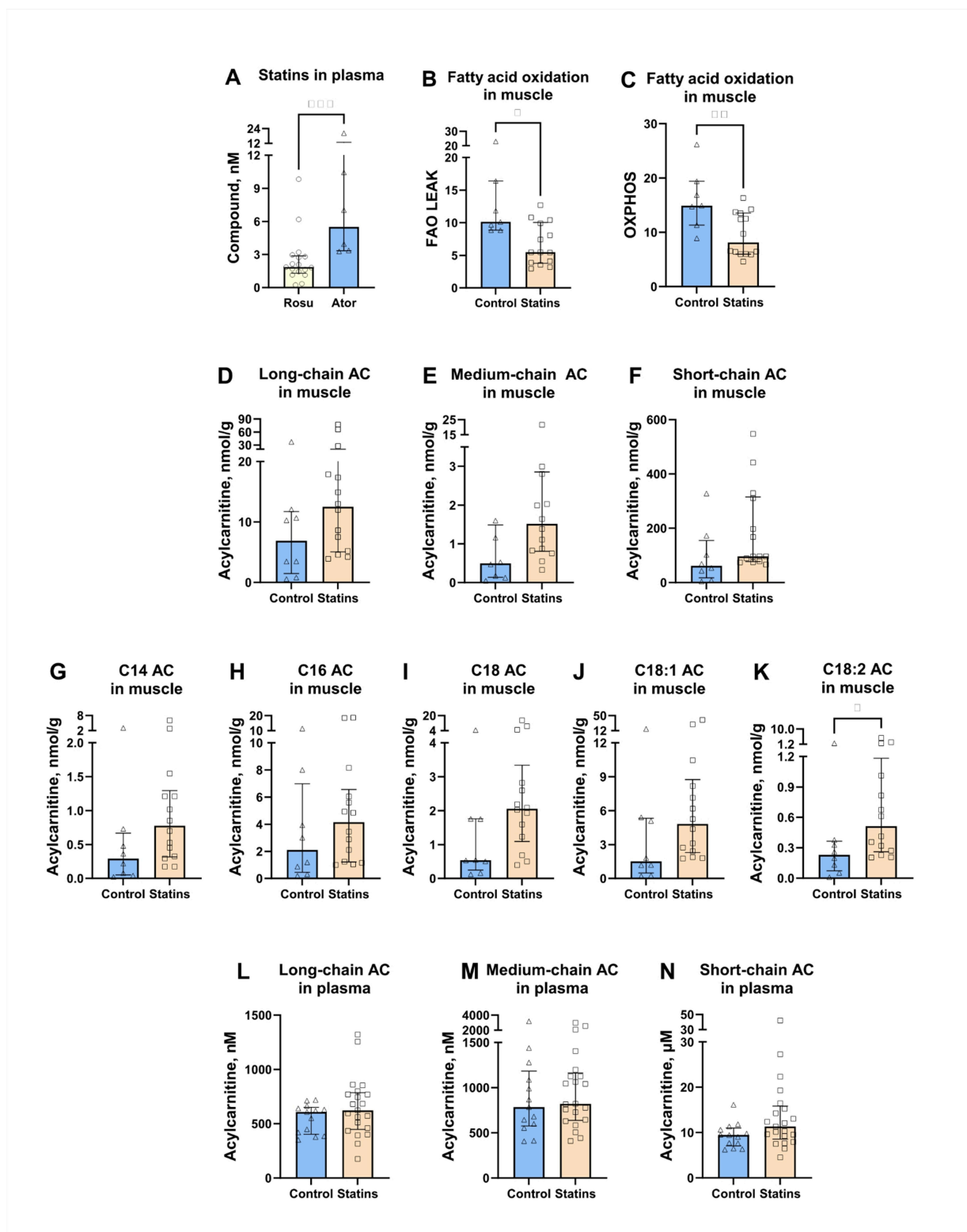


Figure 1

Statin users have a lower fatty acid oxidation (FAO) rate and higher levels of long-chain acylcarnitines in muscle. In statin users, the presence of atorvastatin and rosuvastatin in plasma was confirmed (A). In the muscles of statin users, fatty oxidation was lower than that in controls in the LEAK (B) and OXPHOS (C) states. A lower FAO rate led to the accumulation of long- (D) and medium-chain (E) but not short-chain (F) acylcarnitines. The accumulation of individual C14 (G), C16 (H), C18 (I), C18:1 (J), and C18:2

(K) acylcarnitines was also observed. The concentrations of long- (L), medium- (M), and short-chain (N) acylcarnitines in plasma were similar between the groups. The data are presented as the median levels $\pm$ IQRs in samples from 6 to 21 patients. Statistical significance was assessed using the Mann–Whitney test in all panels. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Abbreviations: AC, acylcarnitine; Rosu, rosuvastatin; Ator, atorvastatin; IQR, interquartile range.

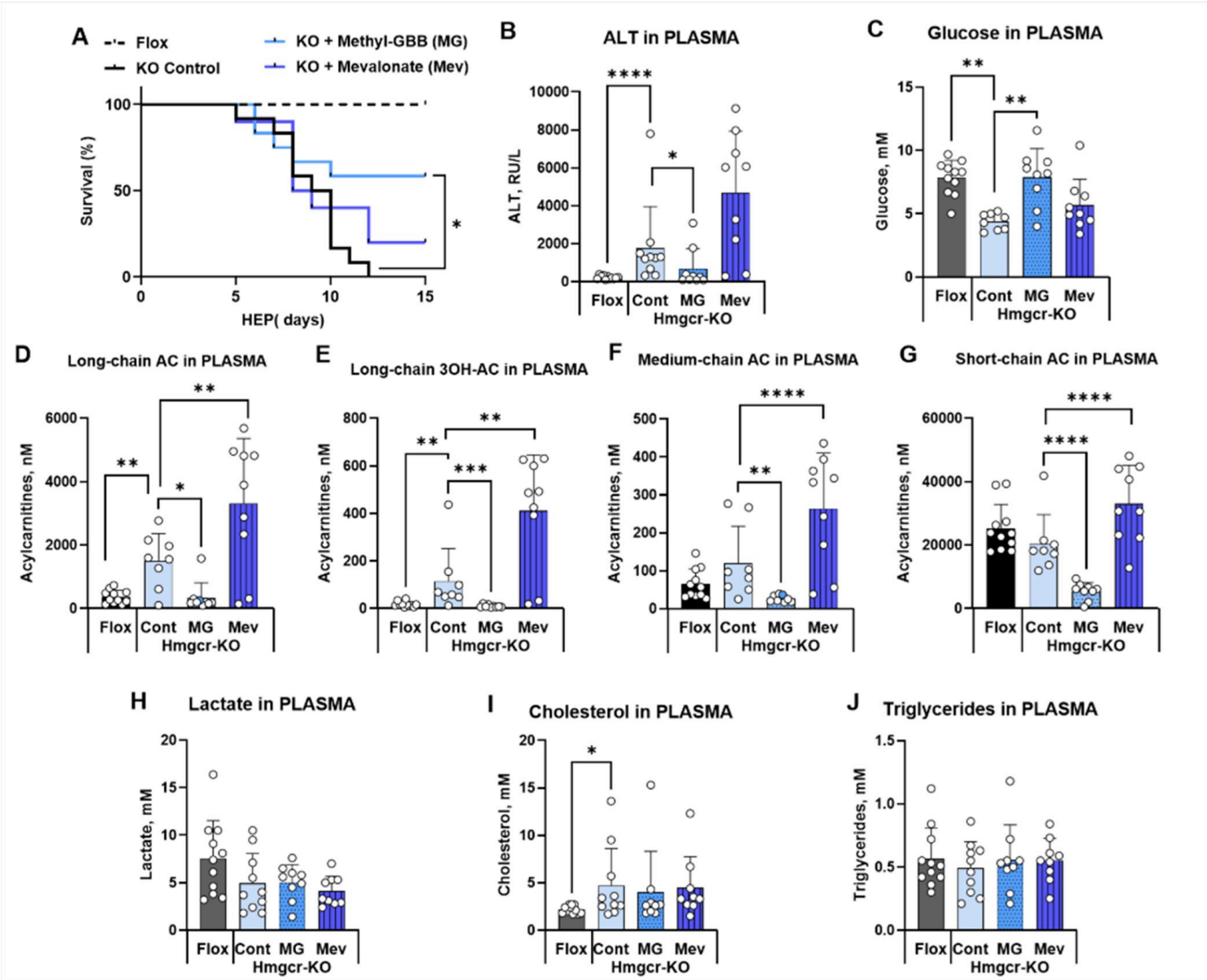


Figure 2

While *Hmgcr* deletion leads to acute lethal liver failure and metabolic disruption, treatment with methyl-GBB can significantly improve survival in male *Hmgcr*-KO mice. (A) According to the Kaplan–Meier plot, a few days after the induction of *Hmgcr*-KO, the mice reached the HEP, and treatment with methyl-GBB significantly increased the survival rate. (B) A substantial increase in plasma ALT levels indicates acute liver failure. Methyl-GBB treatment significantly reduced the elevated ALT level in the KO animals, whereas mevalonate had no effect. (C) A significant decrease in glucose concentration was

observed, whereas methyl-GBB treatment did not lead to any reduction in glucose levels in KO animals. **(D)** Long-chain acylcarnitine (AC) and **(E)** long-chain-3OH AC concentrations were substantially elevated in KO mice, and treatment with methyl-GBB reduced the blood LCAC levels. **(F, G)** Medium- and short-chain AC concentrations were not reliably increased in the KO mice. Biochemical measurements of plasma **(H)** lactate, **(I)** cholesterol, and **(J)** triglyceride levels. Plasma samples were collected from floxed, KO, and KO mice after treatment (methyl-GBB or mevalonate) at the endpoint (Days 7–12). The data are shown as the means $\pm$ SEMs ($n = 8–11$ mice per group). The statistical significance of differences in the survival curves (A) was assessed using the log-rank (Mantel–Cox) test. Pairwise comparisons between the floxed and *Hmgcr*-KO mice in the Control groups (B–J) were performed using the non-parametric Mann–Whitney U test. Differences among the *Hmgcr*-KO mice and treatment groups were evaluated using one-way ANOVA followed by Tukey’s post hoc test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$). *Abbreviations:* HEP, humane endpoint; ALT, alanine transaminase; AC, acylcarnitine; *Hmgcr*-KO, *Hmgcr* knockout; Flox, floxed; Cont, control; MG, methyl-GBB; Mev, mevalonate.

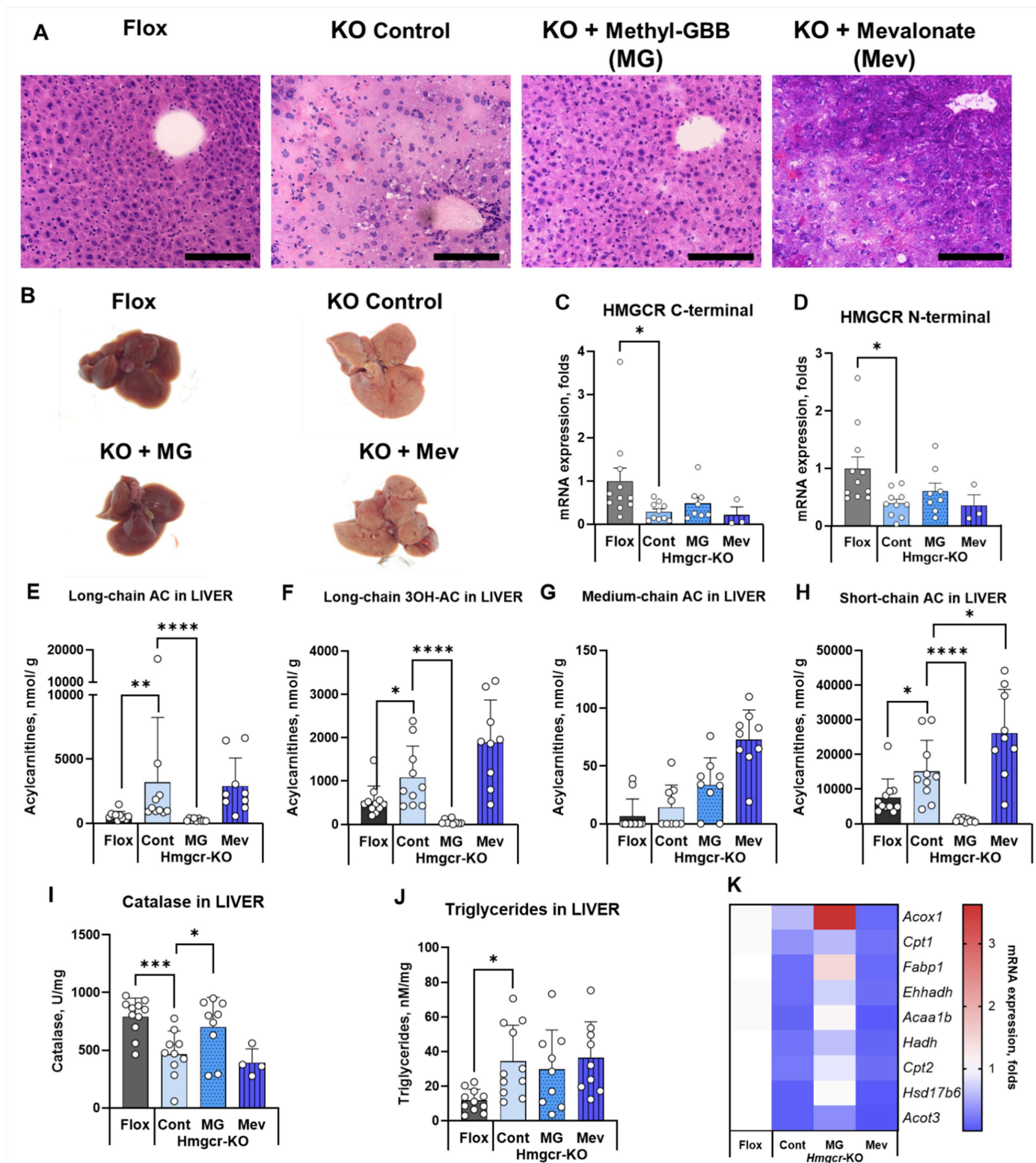


Figure 3

Methyl-GBB therapy protects the liver tissue from *HMGCR* knockout-induced acute injury. (A) Histological analysis of haematoxylin and eosin-stained liver sections (10 μ m, scale bar: 100 μ m) and (B) the macroscopic appearance of a pale liver revealed hepatocellular death in *Hmgcr*-KO mice, whereas methyl-GBB treatment substantially protected the liver tissue from injury. (C, D) The deficiency of the *Hmgcr* gene was confirmed by a decrease in the mRNA levels of the C-terminal and N-terminal

fragments of *Hmgcr*. **(E)**The hepatic long-chain AC, **(F)** long-chain 3OH-AC, and **(H)**short-chain AC concentrations were markedly elevated in KO mice but were reduced by methyl-GBB treatment, whereas no significant changes in **(G)**the medium-chain AC concentrations were observed between groups. Biochemical measurements of **(I)** catalase activity and **(J)** triglyceride concentrations in the liver. **(K)** Heatmap summarizing the average changes in the expression of genes related to fatty acid metabolism and energy management in the liver. Livers were collected from floxed mice, KO mice, and KO mice after treatment (methyl-GBB or mevalonate) at the endpoint (Days 7–12). The data are shown as the means $\pm$ SEMs (n = 8–11 mice per group). Pairwise comparisons between the Flox and *Hmgcr*-KO mice in the Control groups (C–J) were performed using the non-parametric Mann–Whitney U test. Differences among the *Hmgcr*-KO mice and treatment groups were evaluated using one-way ANOVA followed by Tukey's post hoc test (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). *Abbreviations*: LCAC, long-chain acylcarnitines; *Hmgcr*-KO, *Hmgcr* knockout mice; Flox, floxed mice; Cont, saline; MG, methyl-GBB; Mev, mevalonate; *Acox1*, acyl-CoA oxidase 1; *Cpt1*, carnitine palmitoyltransferase 1; *Fabp1*, fatty acid-binding protein 1; *Ehhadh*, enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase; *Acaa1b*, acetyl-CoA acyltransferase 1B; *Hadh*, hydroxyacyl-coenzyme A dehydrogenase; *Cpt2*, carnitine palmitoyltransferase 2; *Hsd17b6*, hydroxysteroid 17-beta dehydrogenase 6; *Acot3*, acyl-coenzyme A thioesterase 3.

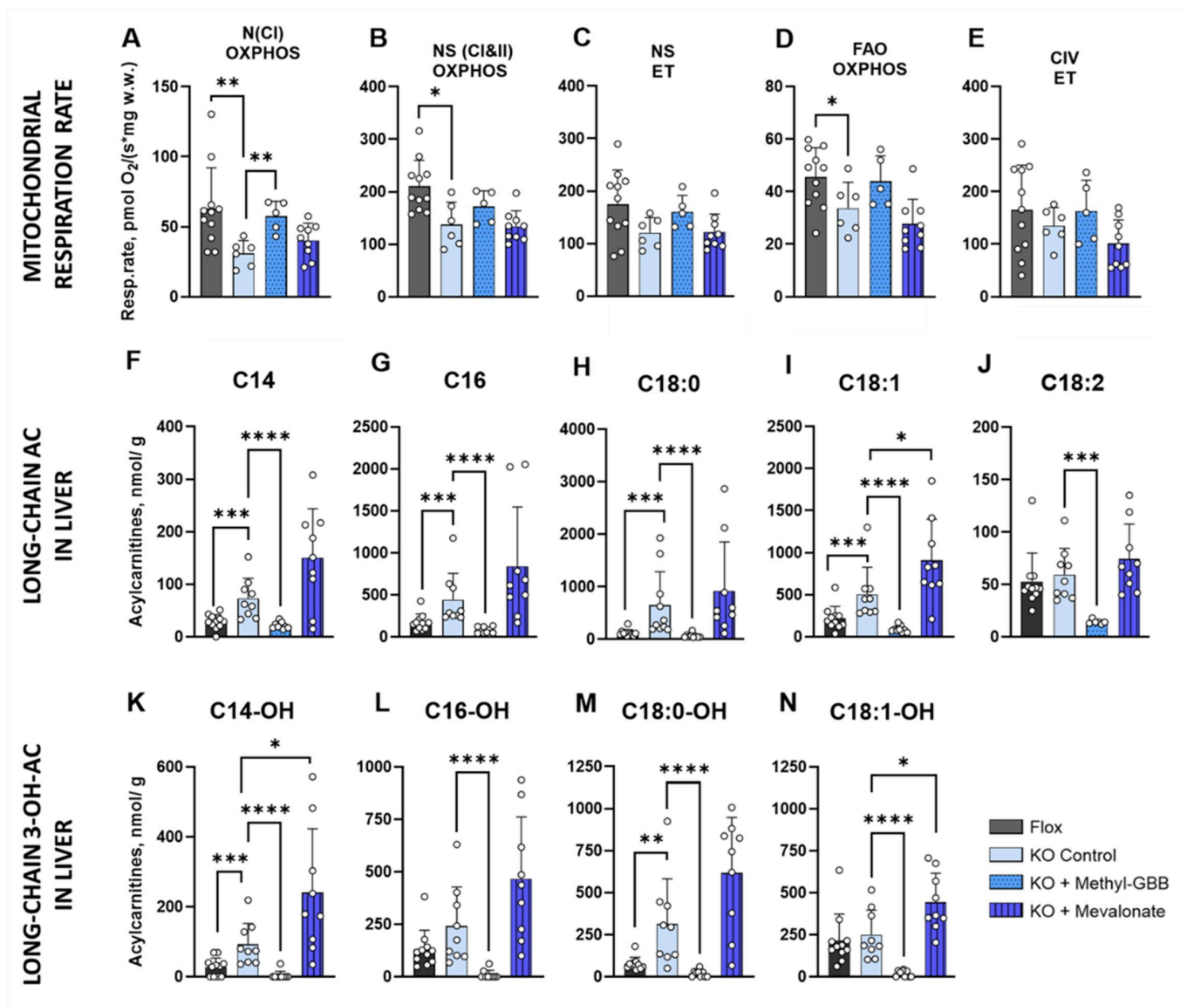


Figure 4

Methyl-GBB protects mitochondrial function by reducing the LCAC concentration in the livers of *Hmgcr*-KO mice. High-resolution respirometry revealed significantly reduced mitochondrial respiration rates based on Complex I and II substrates, as well as on palmitoylcarnitine (fatty acid oxidation), in *Hmgcr*-KO mice (**A, B, D**); moreover, methyl-GBB treatment substantially restored the Complex I-dependent mitochondrial respiratory capacity (**A**). The capacity of the electron transport chain and Complex IV activity remained unaltered in the KO animals (**C, E**). The levels of long-chain acylcarnitines (**F–J**) and long-chain 3-hydroxy-acylcarnitines (**K–N**) were substantially increased in the livers of the KO mice, and the total long-chain acylcarnitine level was very high. Methyl-GBB treatment effectively prevented the hepatic accumulation of long-chain acylcarnitines (**F–N**). Liver tissue samples were collected from the mice at the endpoint (Days 5–12). The data are shown as the means $\pm$ SEMs ($n = 5–11$ mice per group). Pairwise comparisons between the Flox and *Hmgcr*-KO mice in the Control groups

(B–J) were performed using the non-parametric Mann–Whitney U test. Differences among the *Hmgcr*-KO mice and treatment groups were evaluated using one-way ANOVA followed by Tukey's post hoc test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). *Abbreviations*: AC, acylcarnitines; CI, Complex I; CII, Complex II; N, NADH pathway; NS, NADH and succinate pathway; FAO, fatty acid oxidation-dependent pathway; OXPHOS, oxidative phosphorylation; ET, electron transfer; C14, tetradecenoylcarnitine; C14:1-OH, hydroxytetradecenoylcarnitine; C16, hexadecanoylcarnitine; C16-OH, hydroxyhexadecanoylcarnitine; C18:0, octadecanoylcarnitine; C18:1, octadecenoylcarnitine; C18:1-OH, hydroxyoctadecenoylcarnitine; C18:2, octadecadienoylcarnitine; U, uncoupler (carbonyl cyanide 3-chlorophenylhydrazone); LCAC, long-chain acylcarnitines; *Hmgcr*-KO, *Hmgcr* knockout mice; Flox, floxed mice; Cont, control mice; MG, methyl-GBB; Mev, mevalonate.

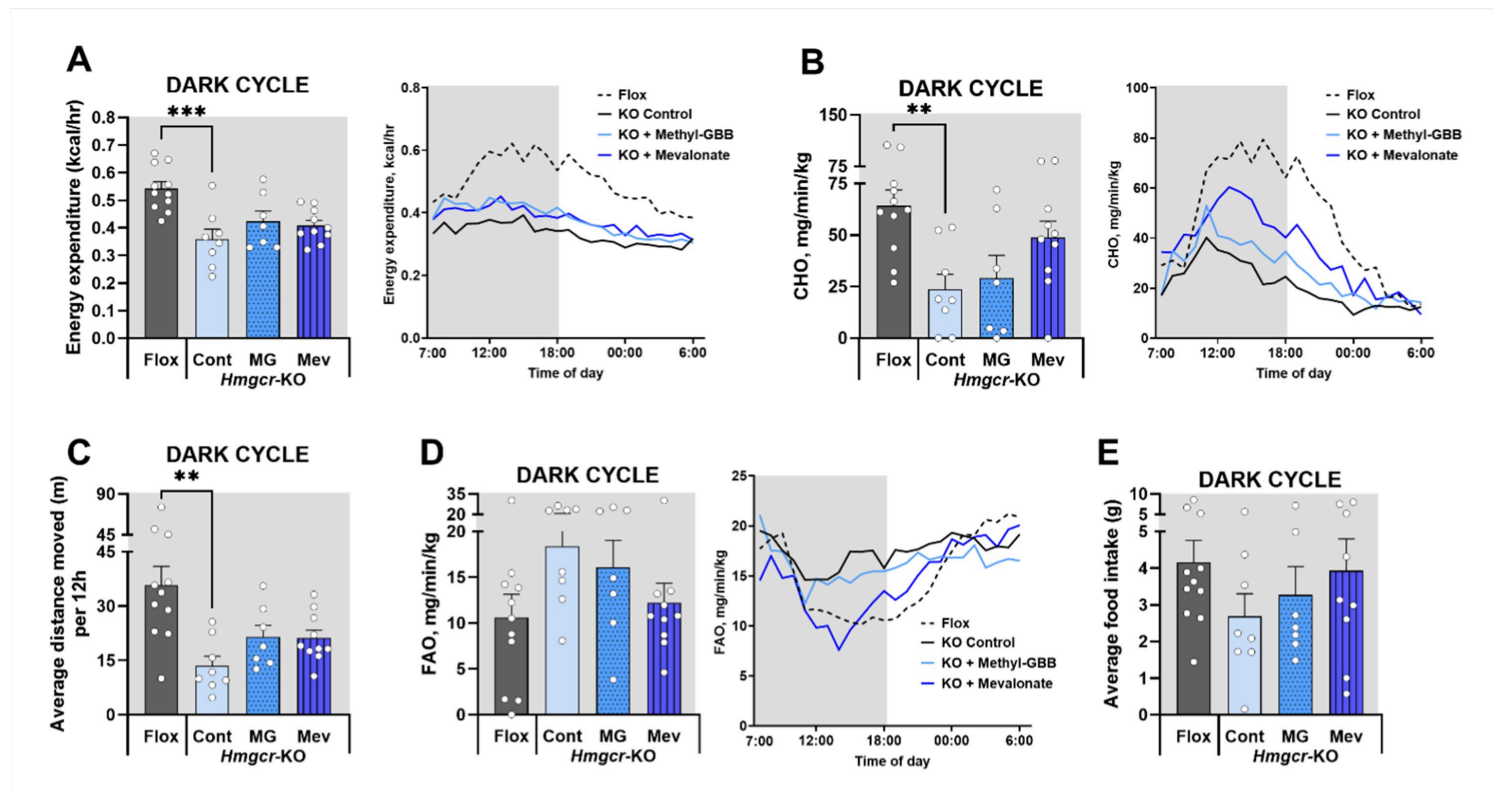


Figure 5

Whole-body metabolic dysfunction and reduced locomotor activity in *Hmgcr*-KO mice during the final 24 hours before the humane endpoint (HEP). Reduced (A) energy expenditure, (B) carbohydrate oxidation, and (C) locomotor activity were observed in *Hmgcr*-KO mice compared with floxed control mice, while (D) whole-body fatty acid oxidation and (E) food intake remained largely unchanged. Methyl-GBB- and mevalonate-treated *Hmgcr*-KO mice displayed metabolic profiles that were broadly similar to those of untreated KO animals. For each parameter, representative time-course measurements are shown as line plots (right panels), while bar plots (left panels) summarize the corresponding average values during the dark cycle (grey shading), during which mice are normally most active and consume the majority of their food. The data are presented as the means $\pm$ SEMs ($n = 8$ – 11 mice per group). ** $P < 0.01$ and *** $P < 0.001$ for Flox mice vs. KO mice in the control group, assessed using the nonparametric

Mann–Whitney test and for KO mice in the control group vs. KO mice in the treatment groups, assessed using the Kruskal–Wallis test. Abbreviations: *Hmgcr*-KO, *Hmgcr*knockout mice; Flox, floxed mice; Cont, control mice; MG, methyl-GBB; Mev, mevalonate.

Supplementary Files

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