SUPPLEMENTARY METHODS

Animals

Forty-nine male C57BL/6 mice (Royan Institute, Isfahan, Iran) were purchased at 4 weeks of age (12 to 14 g of body weight). Mice were housed in the same room for the entire experimental period under the standard conditions (non-barrier) with a 12-hour light-dark cycle (7:00 AM to 7:00 PM) in a controlled temperature (23°C±1°C) and humidity (50% to 60%). All mice had *ad libitum* access to water and food. Body weight was recorded and the cage location was randomly changed weekly. All mice were treated as planned and had no health issues.

Diet

After one habitation week upon arrival, mice were randomly divided into two groups: normal diet (10% fat, 20% protein, 70% carbohydrate; $n=7$) and high-fat diet (HFD; 60% fat, 20% protein, 20% carbohydrate; $n=42$) (Faradamzarin, Isfahan, Iran). These diets were applied for 12 weeks as the first stage of the study. After the first stage, HFD-treated mice were further randomly divided into six groups ($n=7$/group): (1) no treatment (pre-D), (2) green coffee (GC) intake, (3) chlorogenic acid (CGA) intake, (4) exercise training (EX), (5) GC+EX, and (6) CGA+EX. The corresponding treatments were applied for additional 10 weeks under the same diet as the first stage. The sample size ($n=7$/group) was determined using G*Power software for 0.75 of the effect size at a 5% significance level with 90% power [1].

GC tablets (Bonyan Salamat Kasra, Tehran, Iran) contain natural products; in particular, 400 mg of standardized GC bean extract powder containing 2% caffeine and 50% CGA [2]. The analysis of CGA in GC bean extract was performed using a high-performance liquid chromatography-diode array detector gradient system (Agilent 1090 series, Agilent, Santa Clara, CA, USA) (Supplementary Fig. 1). GC (200 mg/kg in 200 μL) or CGA (100 mg/kg in 200 μL) was administered three times per week as a gavage supplement (Sigma-Aldrich, St. Louis, MO, USA) dissolved in water, as previously described [3].

At the end of the second stage, the animals were fasted overnight (12-hour) and were anesthetized by intraperitoneal injection of a cocktail of 10% ketamine (50 mg/kg) and 2% xylazine (10 mg/kg). Blood was taken from the right ventricle, collected in tubes containing ethylene-diamine-tetraacetic acid (EDTA), centrifuged at 2,000 g for 10 minutes to obtain serum, and immediately stored at −80°C. Then, mice were euthanized by cervical dislocation. Liver tissues were immediately obtained and stored at −80°C for further analyses.

Exercise training protocol

The exercise was performed on a rodent treadmill (MazeRouter, Tabriz, Iran) at a moderate intensity [2] for 10 weeks during the second stage of the study. Mice began to run on the treadmill at 17 m/min in the first 2 weeks. Then, the running speed was gradually increased every 2 weeks to reach 23 m/min in the final 2 weeks of the training period. Mice ran for 45 min/day and 5 day/week. Each exercise session was composed of 3 minutes of warm-up, 40 minutes of running, and 2 minutes of recovery. Not exercising mice were also placed on the treadmill but were not committed to running. The exercise was performed between 10:00 and 11:00 AM.

Biochemical analyses

Fasting blood glucose and glucose tolerance tests (GTT) were performed both at the end of the first stage (12th week) and the second stage (22nd week) using a tail prick and glucometer (Alpha TRAK glucometer, Zoetisus, Parsippany-Troy Hills, NJ, USA). For GTT, 6-hour fasted mice were administered 200 μL glucose water solution by nasogastric feeding. Blood glucose levels were measured at 0, 30, 60, 90, and 120 minutes after feeding. Plasma insulin levels were determined using an Ultra-Sensitive Mouse Insulin enzyme-linked immunosorbent assay (ELISA) Kit (80-INSMS-E01, ALPCO, Salem, NH, USA), following the manufacturer's instructions. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured using mouse AST (CSB-E12649m) and ALT (MBS016898) ELISA kits (Abcam, Cambridge, UK), respectively. High-density lipoprotein and low-density lipoprotein levels were determined using a kit (Cat. K613-100, Cell Biolabs Inc., San Diego, CA, USA), according to the manufacturer's protocol. All ELISA assays were performed using an NS-100 Nano Scan Microplate Reader (Hercuvan Lab Systems, Cambridge, UK).

In silico data analysis

To identify candidate genes regulated by HFD, GC, and exercise in the mouse liver, publicly published data from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) were searched and analyzed. The keywords GC, liver, HFD, and endurance training were used to obtain the qualified data. As a result, two studies with accession numbers

GSE53131 and GSE104079 were chosen for further analyses. Raw data from these studies were downloaded, and the initial preprocessors, including background correction, normalization, and data transfer in a logarithmic mode based on 2, were performed using affy and limma packages. The result of the expression matrix was used in the analysis, and information about the probes used in both studies was evaluated to identify candidate genes. Microarray data analysis was performed using the R software version 4.1 (R Foundation for Statistical Computing, Vienna, Austria). The linear model method was used to identify microarray data that differed between groups, and a false discovery rate of <0.05 was considered significant.

RNA isolation and gene expression analysis

RNA was isolated from frozen liver samples using the TRIzol protocol (Thermo Fisher Scientific, Waltham, MA, USA). The concentration and purity of the extracted RNA were assessed using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific). RNA (1,000 ng) with an integrity number ≥7.5 were transcribed into cDNA using a biotech rabbit cDNA Synthesis Kit (biotechrabbit GmbH, Berlin, Germany). Quantitative reverse transcription polymerase chain reaction was performed with Real QPlus 2x Master Mix Green with low ROX (Amplicon, Brighton, UK) using Step One Plus (Applied Biosystems, Waltham, MA, USA). The primers are listed in Supplementary Table 1. Relative gene expression was calculated using the ΔΔCt method.

Protein extraction and immunoblotting analysis

Frozen livers were lysed using TRIzol reagent (Thermo Fisher Scientific), according to the manufacturer's protocol. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts (30 μg) of total protein from each sample were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes. After blocking with 10% skim milk, the membranes were incubated with primary antibodies for 2 hours at room temperature (23°C±1°C). The primary antibodies used were as follows: acyl-CoA synthetase 3 (ACSL3; 1:1,000, sc-166374), acetyl-CoA carboxylase 1 (ACC1; 1:1,000, sc-137104), acetyl-CoA carboxylase 2 (ACC2; 1:1,000, sc-390344), glycerol-3-phosphate acyltransferase 1 (GPAT1; 1:1,000, sc-398135), carnitine palmitoyl transferase 1 (CPT1; 1:1,000, sc-514555), and β-actin (1:1,000, sc-47778) (Santa Cruz Biotechnology, Dallas, TX, USA). The membranes were then incubated for 1 hour at room temperature with the following secondary antibodies: horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG; 1:5,000, P0447) (Agilent) and HRP-conjugated goat anti-rabbit IgG (1:16,000, sc-2301) (Santa Cruz Biotechnology). The band intensity was quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Oil red O staining

The frozen livers were sectioned and washed with phosphate buffer saline, then stained with a hematoxylin solution. As previously described [4], the sections were stained with oil red O (ORO) staining solution after incubation in 60% isopropanol for 10 minutes. Finally, the sections were washed with running water and were observed under am Olympus BX40 microscope (Olympus, Tokyo, Japan). ORO-stained sections were analyzed to quantify lipid content using ImageJ software [5].

Statistical analysis

Data are presented as the mean±standard deviation. Data from the second stage of this study were analyzed using one-way analysis of variance (ANOVA) to determine the statistical differences among groups. All statistical analyses were performed using SigmaPlot statistical software (version 12.5; https://sigmaplot.com/sigmaplot-ng) with Tukey *post hoc* test. The significance level was set at $P<0.05$.

Ethics statements

The animal study protocol was approved by the Ethics Committee of the Royan Institute (ethics code: IR. ACECR. ROYAN. REC. 1399.075). All animals were treated according to the recommendations of the Animal Ethics Committee of the Royan Institute.

SUPPLEMENTARY REFERENCES

1. Faul F, Erdfelder E, Buchner A, Lang AG. Statistical power analyses using G*Power 3.1: tests for correlation and regression analyses. Behav Res Methods 2009;41:1149-60.
2. Abdollahi M, Marandi SM, Ghaedi K, Safaeinejad Z, Kazeminasab F, Shirkhani S, et al. Insulin-related liver pathways and the therapeutic effects of aerobic training, green coffee, and chlorogenic acid supplementation in pre-Diabetic mice. Oxid Med Cell Longev 2022;2022:5318245.
3. Shimoda H, Seki E, Aitani M. Inhibitory effect of green coffee

bean extract on fat accumulation and body weight gain in mice. BMC Complement Altern Med 2006;6:9.
4. Lu D, Xia Q, Yang Z, Gao S, Sun S, Luo X, et al. ENO3 promoted the progression of NASH by negatively regulating ferroptosis via elevation of GPX4 expression and lipid accumulation. Ann Transl Med 2021;9:661.
5. Sozen E, Demirel-Yalciner T, Sari D, Avcilar C, Samanci TF, Ozer NK. Deficiency of SREBP1c modulates autophagy mediated lipid droplet catabolism during oleic acid induced steatosis. Metabol Open 2021;12:100138.