

Mitofusin-2 induced by exercise modifies lipid droplet-mitochondria communication, promoting fatty acid oxidation in male mice with NAFLD

Juan Carlos Bórquez¹, Francisco Díaz-Castro¹, Francisco Pino-de La Fuente¹, Karla Espinoza¹, Ana María Figueroa², Inma Martínez-Ruiz^{3,4}, Vanessa Hernández⁵, Iliana López-Soldado^{3,4}, Raúl Ventura^{3,4}, Joan Carles Domingo³, Marta Bosch⁶, Alba Fajardo⁶, David Sebastian⁷, Alejandra Espinosa^{8,9}, Albert Pol⁶, Antonio Zorzano^{3,5,10}, Víctor Cortés^{2*}, María Isabel Hernández-Alvarez^{3,4,10*}, Rodrigo Troncoso^{1,11,12*}

¹Laboratorio de Investigación en Nutrición y Actividad Física (LABINAF), Instituto de Nutrición y Tecnología de los Alimentos (INTA), Universidad de Chile.

²Department of Nutrition, Diabetes and Metabolism, Pontificia Universidad Católica de Chile.

³Departament de Bioquímica i Biomedicina Molecular, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain.

⁴Institut de Biomedicina de la Universitat de Barcelona IBUB, Barcelona, Spain.

⁵Institute for Research in Biomedicine (IRB Barcelona). The Barcelona Institute of Science and Technology, Barcelona (BIST), Spain.

⁶Cell Compartments and Signaling Group, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

⁷Department of Biochemistry and Physiology, Faculty of Pharmacy and Food Sciences, University of Barcelona, Barcelona, Spain

⁸Escuela de Medicina, Campus San Felipe, Universidad de Valparaíso.

⁹Department of Medical Technology, Faculty of Medicine, University of Chile.

¹⁰CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM).

¹¹Advanced Center for Chronic Diseases (ACCDiS), Universidad de Chile.

¹²Obesity-induced Accelerated Aging (ObAGE), Universidad de Chile

*Correspondence to: rtroncoso@inta.uchile.cl (RT); mihernandez@ub.edu (MIH-A); vcortesm@uc.cl (VC)

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List of Abbreviations: NAFLD, non-alcoholic fatty liver disease; SHS, simple hepatic steatosis; NASH, non-alcoholic steatohepatitis; FAO, fatty acid oxidation; LDs, lipid droplets; PLIN5, perilipin 5; PDM, peridroplet mitochondria; Mfn-2, mitofusin-2; CD, control diet; HFD, high-fat diet; WT, wild-type; L-KO, liver knockout; CM, cytoplasmic mitochondria; CD, control diet; HFD, high-fat diet; MCD, methionine choline-deficient diet; LD-M: Lipid droplet bound to mitochondria; LD-C: Lipid droplet non-bound to mitochondria.

ABSTRACT

Background and aim: The excessive accumulation of lipid droplets (LDs) is a defining characteristic of nonalcoholic fatty liver disease (NAFLD). The interaction between LDs and mitochondria is functionally important for lipid metabolism homeostasis. Exercise improves NAFLD, but it is not known if it has an effect on hepatic LD-mitochondria interactions. Here, we investigated the influence of exercise on LD-mitochondria interactions and its significance in the context of NAFLD.

Approach and Results: Mice were fed high-fat diet (HFD) or HFD-0.1% methionine and choline-deficient diet (MCD) to emulate simple hepatic steatosis or non-alcoholic steatohepatitis, respectively. In both models, aerobic exercise decreased the size of LDs bound to mitochondria and the number of LD-mitochondria contacts. Analysis showed that the effects of exercise on HOMA-IR and liver triglyceride levels were independent of changes in body weight, and a positive correlation was observed between the number of LD-mitochondria contacts and NAFLD severity and with the lipid droplet size bound to mitochondria. Cellular fractionation studies revealed that ATP-coupled respiration and fatty acid oxidation (FAO) were greater in hepatic peridroplet mitochondria (PDM) from HFD-fed exercised mice than from equivalent sedentary mice. Finally, exercise increased FAO and mitofusin-2 abundance exclusively in PDM through a mechanism involving the curvature of mitochondrial membranes and the abundance of saturated lipids. Accordingly, hepatic mitofusin-2 ablation prevented exercise-induced FAO in PDM.

Conclusions: This study demonstrates that aerobic exercise has beneficial effects in murine NAFLD models by lessening the interactions between hepatic LDs and mitochondria, and by decreasing LD size, correlating with a reduced severity of NAFLD. Additionally, aerobic exercise increases FAO in PDM and this process is reliant on Mfn-2 enrichment, which modifies LD-mitochondria communication.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common hepatic disease worldwide, affecting approximately one-third of adults [1]. It is associated with obesity, diabetes, and cardiovascular diseases, increasing morbimortality [2,3]. NAFLD covers a spectrum of liver disorders, from simple hepatic steatosis (SHS) to nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma. NASH is distinguished from SHS by hepatocyte injury, inflammatory infiltration, and collagen deposition. Exercise has been shown to consistently reduce the progression of NAFLD [4–8], but the underlying mechanisms remain unclear. Although some studies indicate that exercise reduces hepatic fat load [9,10], we and others have reported that the metabolic benefits of exercise occur without changes in hepatic triglyceride content, body mass, or liver fat mass [11–14]. Comparable outcomes were noted among individuals with steatohepatitis, wherein exercise improves liver fibrosis in the absence of clinically significant weight loss [15].

Triglycerides accumulate in hepatic lipid droplets (LDs) [16]. These organelles regulate intracellular fatty acid fluxes by storing and releasing FA for oxidation or re-esterification into triglycerides or other complex lipids, thereby preventing cytotoxic events [16,17]. Structurally, LDs contain a neutral lipid core primarily composed of triglycerides, and a superficial monolayer of phospholipids decorated with proteins of various families [17]. The life cycle of LDs involves biogenesis in the endoplasmic reticulum, expansion through local triglyceride synthesis on their surface, and catabolism via lipolysis and lipophagy [16,17]. Hepatic LDs are heterogeneous not only in size and associated proteins, but also in their interaction with other organelles, including mitochondria. It is not yet clear whether the morphological and biochemical properties of LDs contribute to NAFLD progression [18].

Multispectral time-lapse imaging analysis has revealed that mitochondria are the second most common interaction partners of LDs after the endoplasmic reticulum [19]. The physiological significance of LD-mitochondria contacts has been more closely studied in the brown adipose tissue and skeletal muscle tissues. Brown adipose adipocytes have a population of mitochondria tightly bound to LDs, known as peridroplet mitochondria (PDM) [20], which appear to boost triglyceride synthesis rather than promote fatty acid oxidation (FAO) [21]. Conversely, FAO rates are higher in free cytoplasmic mitochondria (CM) than in PDM [21]. The situation is different in skeletal muscle, where LD-mitochondria contacts are associated with higher FAO from LD

1 catabolism and are more abundant in oxidative (type I) than in glycolytic (type II) muscle
2 fibers, both in patients with diabetes and in individuals with normal body weight [22].
3 Notably, engaging in exercise is linked to increased contacts between LDs and
4 mitochondria in the muscles of healthy subjects, which is associated with increased FAO
5 rates [23], indicating that these contacts are dynamic and responsive to metabolic
6 changes. The role of LD-mitochondria interactions in the liver is less clear and results
7 have been mixed. For example, Najt *et al.* have recently demonstrated that PDM promote
8 the expansion of LDs[24], pointing to a lipogenic effect akin to what is observed in BAT
9 [21]. By contrast, others have found that PDM promote hepatic FAO [25].

16 The structural basis of LD-mitochondria contacts depends on protein tethers, but
17 the mechanisms are uncertain. Perilipin 5 (PLIN5) is an LD surface protein whose C-
18 terminal domain is essential for establishing LD-mitochondria contacts in a range of cell
19 types, including hepatocellular cell lines [26,27], and its depletion in murine hepatocytes
20 decreases LD-mitochondria contacts and lowers triglyceride synthesis and FAO[28].
21 Contrastingly, PLIN5 overexpression worsens hepatic steatosis in high fat-fed mice [29].

27 Mitofusin 2 (Mfn-2) is an outer mitochondrial membrane protein involved in
28 mitochondrial dynamics and hepatic lipid metabolism[30,31]. It has been suggested that
29 Mfn-2 has a crucial role in determining LD-mitochondria contacts, as its gene deletion
30 blunts these interactions in brown adipocytes and decreases FAO rates and thermogenic
31 activity [32]. Nevertheless, the role of Mfn-2 in LD-mitochondria interactions in the liver
32 is currently unknown. Of note, Mfn-2 is downregulated in patients with NASH, and its
33 gene deletion specifically in murine liver triggers a NASH-like phenotype and
34 hepatocellular carcinoma [30].

42 The potential impact of exercise on LD-mitochondria contacts in the liver, and the
43 underlying molecular regulatory mechanisms and implications for NAFLD, remain
44 unknown. Here, we studied the effects of aerobic exercise on hepatic LD-to-mitochondria
45 contacts in murine models of SHS and NASH. Our findings reveal that aerobic exercise,
46 in addition to its effects on weight gain, decreases the number of hepatocellular LD-
47 mitochondria contacts in both models, leading to increased FAO and the enrichment of
48 Mfn-2 in PDMs. Mechanistically, we show that Mfn-2 is crucial for exercise-induced
49 FAO in the PDM of mice, and is linked to increased mitochondrial membrane curvature.

MATERIALS AND METHODS

Animals and diets

Animal care and procedures were approved by the Institutional Animal Care and Use Committee (CICUA) of the Universidad de Chile (20422–INT–UCH) and by the Animal Experimentation Committee of the Barcelona Science Park. Experiments were performed in accordance with the European Community Council Directive and the National Institute of Health guidelines for the care and use of laboratory animals. C57BL/6J male mice were housed in a temperature-controlled (20–23°C) room with a 12:12-h light-dark cycle (6 AM to 6 PM) and had *ad libitum* access to food and tap water.

Cohort 1. Forty-six C57BL/6J male mice (4 weeks old) were randomly assigned to a control diet (CD, 20% protein, 70% carbohydrate, and 10% fat in total calories; D12450J, Research Diets, New Brunswick, NJ, USA) or a high-fat diet (HFD, 20% protein, 20% carbohydrate, and 60% fat; D12492, Research Diets) for 8 weeks. The mice were subdivided into the following four groups: CD, CD + exercise, HFD, and HFD + exercise. Animals in the exercise groups were trained for an additional four weeks, maintaining their corresponding diets during this time.

Cohort 2. Ten C57BL/6J male mice (8 weeks old) were randomly assigned to either CD or a methionine choline-deficient diet combined with a high-fat diet (MCD, A06071305 MCD with 45% HFD; Research Diets) and supplemented with 0.1% L-methionine in drinking water, for 3 weeks.

Cohort 3. Fourteen C57BL/6J male mice (8 weeks old) were fed an MCD and were randomly assigned to an exercise or sedentary group for an additional 4 weeks.

Cohort 4: For electron microscopy analysis, three control LoxP (wild-type, WT) and three liver-specific Mfn-2 knockout (Mfn-2 L-KO) C57BL/6J male mice (12 weeks old) were used. Mfn-2 L-KO mice were generated as described[30]. Briefly, Mfn2^{loxP/loxP} mice were provided by Dr. David C. Chan[33] of the Mutant Mouse Regional Resource Center. To generate L-KO mice, albumin (Alb)-Cre mice were crossed with Mfn2^{loxP/loxP} mice. The control and L-KO mice were littermates.

1 Cohort 5: Eighteen male WT and 13 Mfn-2 L-KO mice (12 weeks old) were fed a HFD
2 for 8 weeks. Mice were then subdivided into the following four groups: WT Sedentary
3 (WT Sed), WT Exercise (WT Ex), Mfn-2 L-KO Sedentary (Mfn-2 L-KO Sed), and Mfn-
4 2 LKO Exercise (Mfn-2 L-KO Ex), and were followed for an additional 4 weeks.
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8 Cohort 6: Nineteen C57BL/6J male mice (4 weeks old) were fed an HFD for 8 weeks,
9 after which they were randomly assigned to two groups. One group was injected via the
10 tail vein with an adeno-associated virus serotype 8 (AAV) driven by the cytomegalovirus
11 promoter and containing *PLIN5* cDNA (1×10^3 genome copies/mouse; VectorBuilder
12 Inc., Chicago, IL, USA). The other group was similarly injected with a GFP-encoding
13 AAV (1×10^3 genome copies/mouse; VectorBuilder Inc.) as a control. After injection,
14 the mice were kept sedentary or trained for 4 weeks.
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23 All mice were euthanized by exsanguination under general anesthesia with 5%
24 isoflurane (Zoetis, Parsippany, NJ, USA) after 6 h of fasting. Blood was rapidly drawn
25 from the abdominal vena cava into pre-cooled syringes containing EDTA and stored on
26 ice. Blood was centrifuged (3500 rpm for 10 min, 4°C), and plasma was recovered and
27 frozen (-80°C) until analysis.
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34 **Treadmill exhaustion test and exercise training protocol**

35 All mice in the exercise groups were acclimatized to the treadmill (Columbus
36 Instruments, LE8710; Harvard Apparatus, Holliston, MA, USA) for three days. On the
37 first and second days, mice ran for 10 min at a speed of 0.5 km/h (14 cm/s) with a slope
38 of 0°. On the third day, the mice ran for 10 min at 0.5 km/h (14 cm/s), 5 min at 0.6 km/h
39 (17 cm/s), and 5 min at 0.8 km/h (22 cm/s) with a 0° slope. Following two days of rest,
40 the mice were subjected to a graded exercise test until voluntary exhaustion. The test
41 began at 0.5 km/h (14 cm/s) with a slope of 0° for 5 min, followed by a speed increase of
42 0.1 km/h (3 cm/s) every 3 min until the animal could no longer maintain treadmill speed.
43 The same protocol was followed at the end of the training period.
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54 Exercise training on the treadmill was performed for 4 weeks, 5 times per week for 1 h
55 each session, at 60–65% of the maximal speed reached on the treadmill exhaustion test.
56 Finally, the mice were sacrificed at least 72 h after the last exercise session.
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Grip strength test

The total strength was determined using an all-grip strength test using a force gauge (Force Gauge, Dongguan, China). Mice were placed horizontally over the metal grid and pulled continuously by the tail until they could no longer hold. The procedure was repeated three times for each mouse to obtain a mean value. Strength was determined before and after training. Grip strength was expressed as N (Newtons) and normalized to the body weight (N/g).

Glucose tolerance test

An intraperitoneal glucose tolerance test (GTT) was performed after 12 weeks. Mice were fasted for 6 h with free access to water. A single dose of glucose (2 g/kg body weight dissolved in saline) was administered by intraperitoneal injection, and blood glucose levels were measured in the tail vein at 0, 10, 20, 30, 60, 90, and 120 min using an Accu-Check Performa glucometer (Roche Diagnostics, Mannheim, Germany).

Biochemical analysis

Plasma concentrations of alanine (ALT) and aspartate (AST) transaminase (in U/L), total cholesterol, c-HDL, and triglycerides (all in mg/dL) were measured using a Spotchem II Kenshin-2 kit (77188, Arkay, Kyoto, Japan) in a SpotChem EZ Analyzer (Arkay, Kyoto, Japan). LDL-cholesterol (mg/dL) was determined using the Friedewald formula. Plasma insulin levels were determined using an enzyme-linked immunosorbent assay kit (10-1247-01; Mercodia, Uppsala, Sweden).

Hepatic triglyceride measurements

Levels of mouse liver triglycerides were quantified using the Triglyceride Determination Kit TRO100 with appropriate standards (Sigma-Aldrich, St. Louis, MO). Frozen liver samples were weighed, placed into 2 mL of a chloroform:methanol mixture (2:1, v/v), and incubated for 2 h at room temperature with occasional shaking. Following the addition of 0.2 volumes of water, vortexing, and centrifuging at $2,500 \times g$, the lower phase containing the lipids was collected and dried under vacuum in a rotary evaporator for 5–6 h. The dried pellets were resuspended in the reaction buffer provided in the kit. Results were expressed as mg/g tissue.

Histology analysis

Liver sections were stained with hematoxylin-eosin or Sirius Red. Images were visualized under a light microscope (CX22, Olympus, Tokyo, Japan) and 20× fields were analyzed from the same liver lobe of each animal using Toup View software (ToupTek Photonics, Zhejiang, China). The number and area of lipid droplets were measured with ImageJ version 1.51 (National Institutes of Health, Bethesda, MD, USA). NAFLD score was determined according to Liang *et al* [34]. Sirius red staining was quantified with QuPath software (QuPath developers, University of Edinburgh, UK)[35].

Transmission electron microscopy

Liver samples were processed as described[30]. Briefly, samples were sectioned into small fragments (1 mm) with a razor blade, fixed in 2.5% glutaraldehyde 2% paraformaldehyde solution at 4°C for 2 h, and then washed three times with 0.1 M phosphate buffer. The samples were then treated with 1% osmium tetroxide in 0.1 M phosphate buffer at 4°C for 2 h, washed with highly pure water, and kept overnight in 0.1 M phosphate buffer. Subsequently, the samples were dehydrated at 4°C with shaking through a graded acetone series (50%, 70%, and 90% in highly pure water). Gradual infiltration with Eponate 12 Resin (Ted Pella, Redding, CA, USA) was followed by polymerization of the resin for 48 h at 60°C. Ultrathin sections (50 nm) were obtained using a Leica EM UC6 microtome (Leica, Vienna, Germany) and mounted on 200-mesh copper grids. Tissue sections were stained with 2% uranyl acetate for 30 min, washed with highly pure water, incubated for 5 min with lead citrate, and air-dried. The sections were examined by transmission electron microscopy (Tecnai T12 at 80 kV; Philips. Advanced Microscopy Unit Pontificia Universidad Católica de Chile and Jeol EM J1010 100 kV, CCIT of Universitat de Barcelona). For experiments using HFD, 112 electron micrographs were obtained at 8,200× magnification in a randomized, systematic order; for MCD experiments 135 electron micrographs were obtained at 12,000× magnification. Finally, 28 micrographs of Mfn-2 L-KO, 38 micrographs of WT/Mfn-2 L-KO mice under HFD and Sedentary/Exercise, and 44 micrographs of AAV experiments were obtained at a magnification of 12,000×.

The identification of mitochondria on micrographs was based on the visualization of a spherical or elongated shape in the cytosol, double membrane, and high electron density [36]. LDs were identified by visualizing circular grayish-white structures with a

diffuse rim (absence of a clearly visible membrane) and irregular membranes, as described [22].

Liver analyses were performed using Fiji/ImageJ software (National Institutes of Health). Because the LD-mitochondria interaction is described as adherence rather than proximity [22,37], physical contact between the edge of the LD surface and the outer membrane of the mitochondrion by electron microscopy was considered as an LD-mitochondria interaction. In addition, high electron density is often observed at the contact sites between the two organelles. Data are presented as the LD number of contacts relative to the total number of mitochondria or LDs. The sizes of the mitochondria and LDs were calculated as the mean area of each micrograph. The aspect ratio (length-to-width ratio) and number of mitochondria were calculated for each micrograph using Fiji/ImageJ software (particle analysis). Mitochondrial density was calculated as the sum of the area of each mitochondrion divided by the total area of the electron micrograph.

Lipid droplet isolation

Hepatic LDs were isolated as described [38]. Briefly, isolated liver was perfused with 0.9% NaCl and 0.1% EDTA solution, placed in a Petri dish, chopped with a scalpel for 2 min, and then transferred to a Dounce tissue grinder at a ratio of 1 g of tissue to 3 mL of homogenization buffer (25 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM EDTA, and 5 mM EGTA). After six up-and-down strokes at 200 rpm (RZR 2020, Heidolph Instruments, Schabach, Germany), the homogenate was centrifuged at 500 g for 10 min at 4°C. Subsequently, 2.5 mL of the resulting post-nuclei supernatant was mixed with an equal volume of 2.5 M sucrose and placed at the bottom of a sucrose step gradient of 25%, 15%, 10%, and 5% (w/v) sucrose in homogenization buffer, with an additional top layer of 25 mM Tris-HCl, pH 7.5, 1 mM EDTA and 5 mM EGTA, and was then centrifuged at 12,000 ×g for 1 h at 4°C (SW-41Ti rotor, Beckman Coulter, Pasadena, CA, USA). Six or seven fractions were collected from the top. To purify LDs, the LD fraction at the top of the gradient was recovered and concentrated by re-floating LDs at 16,000 × g for 10 min at 4°C. The lower phase containing the excess buffer was removed by aspiration with a syringe, and four volumes of ice-cold acetone were then added to precipitate proteins, with incubation for 48 h at -20°C. The samples were centrifuged at 16,000 × g for 10 min at 4°C and the pellet was washed with cold acetone three times, air-dried, and reconstituted with 10 mM Tris-HCl, pH 7.5). After sonication, the protein concentration

was quantified using the Novagen BSA assay (71285-3, Sigma-Aldrich) (Supplementary Fig. S1).

Isolated mitochondria subpopulation

Cytoplasmic mitochondria and PDM were isolated as described[21,37,39]. Briefly, approximately ~1.5 g of liver was washed with PBS, placed on a Petri dish, chopped with a scalpel, transferred to a Dounce tissue grinder, and treated with 7 mL of mitochondrial isolation buffer + BSA (50 mM sucrose, 5 mM HEPES, 2 mM EGTA, 2 mg/200 mL fatty acid-free BSA). After 10 up-down strokes at 200 rpm in a homogenizer (RZR 2020, Heidolph Instruments), the homogenate (aliquots of 200 μ L) was centrifuged at $900 \times g$ for 11 min at 4°C. The resulting lipid and aqueous phases were separated and centrifugation was repeated. The lipid and aqueous phases were recovered from each tube (200 μ L of each phase) and centrifuged at $9000 \times g$ for 18 min at 4°C (Type 90 Ti fixed-angle rotor; Centrifuge L-90K ultracentrifuge, Beckman Coulter). After recovering the lipid phase (stripping the fat phase, 200 μ L) from one tube and the aqueous phase (cytosol, 200 μ L) from the other tube, the resulting pellets from both tubes (PDM and CM) were resuspended in mitochondrial isolation buffer without BSA. Aliquots stored along the process were analyzed by immunoblotting to determine the purity of the isolated mitochondrial subpopulations (Supplementary Fig. S2).

Oxygen consumption assessment in an isolated mitochondrial subpopulation

The CM and PDM were resuspended in Mitobuffer cells (50 mM sucrose, 5 mM HEPES, and 2 mM EGTA). The suspension was placed in a chamber coupled to a Clark electrode (Oxygraph; Hansatech Instruments, Norfolk, UK). Oxygen levels were recorded at 3-min intervals under baseline (5 mM glutamate/2.5 mM malate), ATP-coupled (150 μ M ADP), and non-ATP-associated (1 μ M oligomycin, 75351, Sigma-Aldrich) conditions. Oxygen consumption rates were calculated and normalized to the protein content using the Novagen BSA assay. The results are shown as fold changes relative to the baseline CM condition.

Fatty acid oxidation

Oleate oxidation to CO₂ was measured in CM and PDM as described[30,40]. Briefly, buffer MKRH (NaCl 115 mM, KCl 2.6 mM, KH₂PO₄ 1.2 mM, NaHCO₃ 10 mM, HEPES

10 mM, ATP 5 mM, 1 mM NAD⁺, L-carnitine, CoA 0.1 mM, malate 0.5 mM, cytochrome C 25 μM) was continuously gassed with 95% O₂, 5% CO₂. Isolated mitochondria were then incubated for 180 min at 37°C with 1 mCi/ml D-[U]-¹⁴C-Oleate (Amersham Biosciences) in MKRH buffer. The test plates were hermetically sealed with parafilm, with the center well containing a piece of filter paper saturated with 200 mL of 1M KOH. At the end of the incubation, the solution was acidified with 0.3 mL of 0.5 N H₂SO₄ and the gaseous ¹⁴CO₂ released after the acidification was trapped in the filter paper. The vials were incubated at 37°C for 60 min and the filter papers were removed and transferred to vials for liquid scintillation counting.

Western blotting

Whole livers were lysed with T-PER lysis buffer (78510; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with commercial phosphatase and protease inhibitors (04906845001 and 11836153001, Roche, Basel, Switzerland) and then centrifuged at 1,000 × g for 10 min at 4°C. The protein content of the supernatant, the LD fractions, and the hepatic mitochondrial subpopulation was measured using the Novagen BCA assay and denatured in SDS buffer. In total, 30–50 μg of proteins were separated by SDS–PAGE (10% polyacrylamide), electrotransferred onto PVDF membranes, and blocked with 5% nonfat milk in 0.1% v/v Tween 20 Tris-buffered saline (pH 7.6). Membranes were incubated in 0.1% v/v Tween 20 Tris-buffered saline with 1:1000 primary antibodies against PLIN5 (1:1000, 26951-1-AP, Proteintech, Rosemont, IL, USA), mtHsp70 (1:1000, #MA3-028, Thermo Fisher Scientific), ATGL (1:1000, sc-365278, Santa Cruz Biotechnology, Santa Cruz, CA, USA), OXPHOS (1:1000, ab110413, Abcam, Cambridge, UK), Mfn-2 (1:1000, ab56889, Abcam), vinculin (1:1000, sc-73614, Santa Cruz Biotechnology), TOM20 (1:1000, D8T4N, Cell Signaling Technology, Danvers, MA, USA), TIM23 (1:1000, sc-514463, Santa Cruz Biotechnology), and porin (1:1000, sc-390996, Santa Cruz Biotechnology) at 4°C overnight, and then re-blotted with horseradish peroxidase-linked secondary antibody (1:1000, Calbiochem, San Diego, CA, USA). Finally, the bioluminescent signals were detected using the ECL-plus reagent (DW1029, Biological Industries Ltd., Beit Haemek, Israel) and a C-DiGit Blot Scanner (LI-COR Inc., Lincoln, NE, USA). Bands were analyzed using Fiji/ImageJ software and quantified using scanning densitometry.

Cloning, expression, and purification of Mfn-2 (1-613)

The cloning, expression, and purification of Mfn-2 (1-613) from *Escherichia coli* was performed as described[30]. Briefly, the construct was amplified with KOD polymerase (Novagen) using the plasmid pCDNA3hMfn-2 as a template for Mfn-2 and the following primer pairs to add the extensions necessary for cloning: Mfn-2 (1-613) Fwd 5'-ATCATCACAGCAGCGGCatgtccctgctcttctc-3 and Mfn-2 (1-613) Rev 5'-ATGGTCTAGAAAGCTTTAcctggatgtcaaggaggccag-3.'

Lipid Strip Binding assays

Binding to Lipid Strip assays was performed as described[30]. Briefly, membrane lipid strips (P-6002 and P-6003) were obtained from Echelon Biosciences (Salt Lake City, UT, USA). The membrane was blocked with 3% fatty acid-free BSA in TBS-Tween 0.1% and gently agitated overnight at 4°C. After discarding the blocking solution, the membrane was incubated with 10 µg/mL of protein at room temperature for 1 h. The protein solution was then discarded and the membrane was washed for ~8 min with TBS-Tween 0.1% (this step was repeated 4–5 times). The membrane was then incubated with a 1:1000 dilution of primary antibody in 3% fatty acid-free BSA TBS-Tween 0.1% and agitated gently for 1 h at room temperature. The antibody solution was discarded and the membrane was washed for ~8 min with TBS-Tween 0.1% (this step was repeated 4–5 times). The membrane was then incubated with a 1:25,000 dilution of secondary antibody in 3% fatty acid-free BSA TBS-Tween 0.1% and agitated gently for 1 h at room temperature. The secondary antibody solution was discarded and the membrane was washed for ~8 min with TBS-Tween 0.1% (this step was repeated 4–5 times). The binding of proteins to lipids was detected using chemiluminescence.

Fatty acid composition analysis

The fatty acid composition of isolated mitochondria was analyzed using GC–MS as methyl esters after a methylation reaction, as described [41]. Briefly, GC analysis was performed on a Shimadzu GCMS-QP2010 Plus gas chromatograph/mass spectrometer with an AOC-20i autoinjector and AOC-20s autosampler (Shimadzu, Kyoto, Japan) on a Suprawax-280 column (Teknokroma Analitica SA, Barcelona, Spain). Fatty acid methyl esters were identified through mass spectral studies and by comparing the FAME elution pattern and relative retention times with a reference FAME mixture (GLC-744 Nu-Chek

Prep. Inc., Elysian MN, USA). The results were expressed in relative amounts (percentage molar of total fatty acids) [41].

Data representation and statistical analysis

To represent the data of variable populations (mitochondria and LDs), we used SuperPlots, which combines the representation of the variability of mitochondrial and LDs populations with the median value per animal [42]. Two-way analysis of variance (ANOVA) or analysis of covariance (ANCOVA) was used for statistical analysis, followed by Tukey's post-hoc test for multiple comparisons (when group differences and/or interactions were significant). Body weight values were used as a covariate in ANCOVA analysis. Student's t-test and Pearson's correlation coefficient were also used as required. Results are expressed as the mean $\pm$ standard error of the mean (SEM), and statistical significance was set at $p < 0.05$. Statistical analyses were performed using GraphPad Prism 9 software (La Jolla, CA, USA).

RESULTS

Exercise improves physical capacity and alleviates the severity of NAFLD in HFD-fed male mice

We investigated the effects of aerobic exercise on NAFLD using a murine model of SHS. C57BL/6J WT male mice were placed on a CD or HFD for 8 weeks, after which time they were randomized to remain sedentary or subjected to a moderate-intensity aerobic exercise program (Ex) on a treadmill five times weekly for 4 weeks (Fig. 1A). As expected, body weight and calorie intake were greater in HFD mice than in CD mice (Supplementary Fig. 3A-D). Moreover, HFD mice developed glucose intolerance (Fig. 1B,C) and insulin resistance (Supplementary Fig. 3E,F), which was accompanied by elevated levels of total cholesterol, c-LDL, c-HDL (Fig. 1D; Supplementary Fig. 3G,H), triglycerides (Fig. 1E), and liver transaminases (Fig. 1F). Exercise capacity was lower in HFD mice than in CD mice, as indicated by the lower maximal speed in the treadmill test (Fig. 1A), as was grip strength (Supplementary Fig. 3I). In addition, epididymal white adipose tissue (eWAT) weight was greater in HFD mice than in CD mice (Supplementary Fig. 4A-C).

We next focused on the effects of aerobic exercise on muscle function, metabolic regulation, and NAFLD severity. Although calorie intake was equivalent in HFD and

HFD+Ex mice (Supplementary Fig. 3D), exercise reversed and improved the HFD-induced decreases in maximal speed (Fig. 1A) and moderately improved grip strength (Supplementary Fig. 3E). Supporting the modest changes associated with this type of exercise, no differences were seen in gastrocnemius+soleus weight (Supplementary Fig. 4D), but muscle fiber size was slightly and significantly greater in HFD+Ex mice than in HFD sedentary mice (Supplementary Fig. 4E,F). Glucose intolerance and insulin resistance were also improved by exercise in HFD mice (Supplementary Fig. 3E,F), and this was accompanied by lower levels of total cholesterol (Fig. 1D), triglycerides (Fig. 1E) and liver transaminases (Fig. 1F). Although exercise had no effect on eWAT weight in mice on HFD (Supplementary Fig. 4A), the average adipocyte size was significantly lower in exercised mice than in equivalent sedentary mice (Supplementary Fig. 4B,C).

Exercise decreases hepatic LD size in HFD-fed mice but does not affect mitochondrial morphology

Liver weight was significantly lower in HFD+Ex mice than in sedentary HFD mice (Supplementary Fig. 5). Analysis of liver histology showed that HFD+Ex mice exhibited a predominantly microvesicular steatosis pattern as compared with sedentary HFD mice (Fig. 2A), which was concordant with a greater number and smaller size of LDs (Fig. 2B,C). Large LDs are a known morphological feature of NAFLD and are associated with disease severity [43]. To study the histopathology of NAFLD, we used a validated rodent score system [34]. We observed that exercise decreased the NAFLD score (Fig. 2D), with no effects on the intrahepatic triglycerides (Fig. 2E). Overall, the results suggest that the beneficial effects of exercise on circulating transaminases and insulin resistance are associated with a reduced size and increased number of hepatic LDs, irrespective of the total hepatic TG load, thus contributing to diminishing the severity of NAFLD.

The observed results of exercise could be due to changes in body weight. As HFD+Ex mice had a lower final body weight than sedentary HFD mice, we performed ANCOVA to control for a potential effect of body weight gain on the studied variables. Analysis revealed that changes in body weight could explain the effect of exercise on NAFLD score (slope, $p=0.941$, Supplementary Fig. 5A), glucose intolerance (slope, $p=0.662$, Supplementary Fig. 6B), total serum cholesterol (slope, $p=0.772$, Supplementary Fig. 5C), serum triglycerides (slope, $p=0.8145$, Supplementary Fig. 6D), serum ALT transaminase (slope, $p=0.189$ Supplementary Fig. 6E), and serum AST transaminase

(slope, $p=0.709$, Supplementary Fig. 6F). Therefore, the impact of exercise on body weight in part explains the improvement in NAFLD. However, the effect of exercise was independent of body weight in HOMA-IR (slope, $p=0.035$, Fig. 2F) and liver triglycerides (slope, $p=0.035$, Fig. 2G), and was close to significance for fasting glycemia (slope, $p=0.061$, Fig. 2H).

Because abnormal mitochondrial morphology and function have been linked to increased NAFLD severity [44], we analyzed the total number, size, aspect ratio, and density (percentage of the area of the liver occupied with mitochondria) of total mitochondria in exercised and sedentary HFD mice. However, we found no changes in these parameters between groups (Fig. 2I,J). We next focused on mitochondrial ultrastructure, particularly interorganelle contacts between LDs and mitochondria, given their potential involvement in hepatic lipid metabolism.

Exercise decreases the number of LD-mitochondria contacts in hepatocytes in association with diminished NAFLD severity

LD-mitochondria interactions have been described as adherent contacts rather than simply displaying close proximity [22,24,37]. We thus examined the contacts between the LD surface and the outer mitochondrial membrane using transmission electron microscopy. We observed that the number of LD-mitochondria contacts (i.e., the number of mitochondria attached to LDs) in the liver was higher in HFD mice than in CD mice, and this increase was greater than expected by the sole accumulation of LDs in steatotic livers (Fig. 3A,B). Notably, exercise significantly decreased the number of LD-mitochondria contacts in HFD mice (Fig. 3A–C). Of note, the number of LD-mitochondria interactions directly correlated with the NAFLD score (Fig. 3D) and with ALT transaminase levels (Fig. 3E). These results suggest that there is a selective increase in LD-mitochondria contacts during hepatic steatosis, and that exercise decreases the number of these interactions in hepatocytes, correlating with lower disease severity.

Several proteins have been identified as mediators of LD-mitochondria inter-organelle interactions in various cell types, of which PLIN5 is the best characterized [45]. Consistent with the ultrastructural findings, the hepatic level of PLIN5 was significantly lower in HFD+Ex mice than in sedentary HFD mice (Supplementary Fig. 7A). To test whether the decrease in the number of LD-mitochondria contacts induced by exercise is due to a decrease in LDs, we normalized the contacts by the number of LDs.

Although the functional implications of LD-mitochondria contacts are still controversial, they may promote lipogenesis in brown adipocytes [21]. We noted that the size of the hepatic LDs was greater when bound to mitochondria (LD-M) than when non-bound to mitochondria (LD-C) (Fig. 3F,G). Under HFD, the size of LD-M was significantly lower in exercised mice than in sedentary mice (Fig. 3F,G). We noted a minor albeit non-significant effect of exercise on LD-C size, suggesting the specific action of exercise on LDs that are physically bound to mitochondria. In accord with these observations, we observed a positive correlation between LD-M size and the number of LD-mitochondria interactions (Fig. 3H), suggesting that LD-mitochondria interactions promote LD expansion in the context of HFD-induced hepatic steatosis. These findings support the hypothesis that LD-mitochondria contacts promote LD accumulation in the liver and that exercise alleviates NAFLD severity by decreasing the number of LD-mitochondria interactions.

We next characterized the morphological and functional features of mitochondria tightly bound to LDs (i.e., PDM) and CM. As the abundance of LDs is very low in CD-fed mice (Fig. 2A,B), the isolation of PDM was challenging, and we instead focused on HFD-fed mice. Under this condition, PDM were found in a significantly lower proportion than CM in both HFD and HFD+Ex groups (Fig. 4A,B). Notably, the morphology of PDM was significantly more elongated in exercised mice than in sedentary mice (Fig. 4C). We isolated PDM and CM from the livers of mice to examine the molecular and functional differences between the two mitochondria subpopulations. We observed that the levels of complex I (NADH: ubiquinone oxidoreductase subunit B8 protein) were lower in PDM than in CM, irrespective of the exercise status of the animals (Fig. 4D,E). No differences were found in the protein levels of other mitochondrial electron transport complexes between PDM from sedentary and exercised mice (Fig. 4D,E). Nevertheless, the ATP synthesis-coupled respiration rate was higher in PDM from exercised mice than from sedentary mice (3.28 O₂ nmol/min/mg protein and 1.29 O₂ nmol/min/mg protein, respectively) (Fig. 4F). ATP synthesis-coupled respiration in CM was also higher in the exercised mice than in sedentary mice (2.31 O₂ nmol/min/mg protein vs. 1.09 O₂ nmol/min/mg protein), although the absolute mitochondrial respiration rates were lower when compared with those of the PDM (Fig. 4F). These results indicate that exercise increases ATP synthesis-coupled respiration rates in both PDM and CM in the liver of HFD-fed mice and that this functional change is not dependent on changes in the abundance of mitochondrial electron transport complexes.

Hepatic LD-mitochondria contacts are elevated in an MCD-induced NASH model

To assess the role of LD-mitochondria in a more severe model of NAFLD, mice were fed an MCD supplemented with 0.1% methionine in the drinking water to mimic NASH (Fig. 5A). No changes in body weight were observed between MCD-fed mice and CD-fed mice after 3 weeks of diet (Fig. 5B); however, LD accumulation (Fig. 5C–E), NAFLD score (Fig. 5F) and collagen deposition in the liver (Fig. 5G) were all significantly higher in MCD mice than in CD mice. At the ultrastructural level, the number of LD-mitochondria contacts was higher in MCD mice than in CD mice normalized by the number of mitochondria (Fig. 5I). Notably, when the LD-mitochondria contacts were normalized by the LD number, the proportion of mitochondria-associated with LDs was even greater than that seen in HFD-fed mice (Fig. 3C), likely due to the larger size of LDs found under MCD, which frequently interacted with more than one mitochondrion (Fig. 5H). However, the mitochondrial morphology in MCD mice was abnormal when compared with mitochondria in CD mice, with a smaller size and ratio between length and width, and a lower density (Fig. 5K). Also, PLIN5 levels were higher in MCD mice than in CD mice in both whole liver lysate (Supplementary Fig. 7B) and isolated LDs (Supplementary Fig. 7C), suggesting that the increased abundance of this protein tether in NASH promotes a greater number of LD-mitochondria contacts.

Exercise decreases hepatic LD-mitochondria contacts, liver inflammatory foci and collagen deposition in the MCD-induced NASH model.

We next assessed the effect of exercise on the severity of NASH and hepatic LD-mitochondria contacts. To do this, mice were fed an MCD for 4 weeks and were randomly designated to sedentary or exercised (MCD+Ex) groups from the beginning of the diet (to ensure a similar duration of 4 weeks of exercise as in the HFD study) (Fig. 6A). Aerobic exercise had no effect on body weight gain (Supplementary Fig. 8A), food intake (Supplementary Fig. 8B), maximal speed (Supplementary Fig. 8C), fasting insulin (Supplementary Fig. 8D), or transaminases levels (Supplementary Fig. 8E,F). However, the exercise group had a slightly lower NAFLD score than the sedentary group (Fig. 6B,C), accompanied by a marked decrease in the presence of inflammatory foci (Fig. 6B,D) and less collagen deposition (Fig. 6E) and microvesicular hepatic steatosis (Fig. 6B), which might be explained by the greater number of smaller-sized LDs (Fig. 6F,G), but without differences in hepatic triglyceride content between the two groups (Fig. 6H).

Moreover, exercised mice had a higher number and density of mitochondria (Fig. 6I,J). These results suggest that exercise ameliorates hepatic fibrosis in the NASH model and decreases the size of hepatic LDs, but increases their total number.

Similar to our findings in HFD-fed mice, aerobic exercise decreased the number of LD-mitochondria contacts in MCD mice (Fig. 7A–C). Concordant with these findings, exercise decreased PLIN5 protein levels in whole-liver lysates (Supplementary Fig. 7D), which is also similar to that seen in HFD-fed mice (Supplementary Fig. 7A). At the ultrastructural level, exercise decreased the size of LD-M in MCD mice (Fig. 7D). Notably, mice on the MCD had larger LDs than mice on the HFD ($0.094 \mu\text{m}^2$ mean MCD vs. $0.068 \mu\text{m}^2$ mean HFD; compare Fig. 7D with Fig. 3G). As in HFD-fed mice, mitochondria were markedly more elongated in PDM than in CM in MCD-fed mice (Fig. 7E), and the size of the LDs bound to mitochondria directly correlated with the number of LD-mitochondria contacts (Fig. 7F). Importantly, however, exercise had no effect on the morphology of PDM in MCD-fed mice (Fig. 7F), which contrasts with the findings under HFD (Fig. 4C). Overall the data indicate that exercise decreases LD-mitochondria contacts and the size of LDs bound to mitochondria in the MCD model of NASH, but does not change the elongated morphology of PDM.

Increasing the number of LD-mitochondria contacts via PLIN5 overexpression fails to modify the effects of exercise on NAFLD severity.

To test whether the effects of exercise on mitochondrial morphology and NAFLD severity depend on the reduction of LD-mitochondria interactions in hepatocytes, we overexpressed PLIN5, which is the best-characterized protein involved in LD-mitochondria and is decreased in the livers of exercised HFD and MCD mice. To do this, mice were placed on HFD for 8 weeks and then exercised for an additional 4 weeks. At week 8, animals were randomly assigned for transduction with a recombinant AAV encoding either PLIN5 or GFP (Supplementary Fig. 9A). As expected, PLIN5 levels were higher in the liver in the HFD+Ex+AAV-PLIN5 group than in the HFD+Ex+AAV-GFP group, but not in the other tissues (Supplementary Fig. 9B,C), indicating specific hepatic AAV transduction. Similarly, GFP protein was exclusively detected in the liver in the HFD+Ex+AAV-GFP group (Supplementary Fig. 9B). PLIN5 overexpression increased LD-mitochondria interactions in the liver (Supplementary Fig. 9D,E), but had no effect on body weight, calorie intake, liver weight, or maximum speed in the treadmill test (Supplementary Figure 9F–I). Furthermore, no significant differences were observed in

the GTT, fasting glycemia, fasting insulin levels, HOMA-IR, total cholesterol, c-HDL, c-LDL, serum triglycerides, liver transaminases, or hepatic steatosis (Supplementary Fig. 9J–T). These results suggest that PLIN5 does not contribute to the effectiveness of exercise in mitigating NAFLD severity, pointing to other factors beyond the physical contact between LDs and mitochondria involved in the mechanisms underlying the effects of exercise on NAFLD.

Exercise increases fatty acid oxidation in NAFLD by enriching Mitofusin-2 in peridroplet mitochondria.

Mfn-2 participates in mitochondrial dynamics by promoting fusion[31], and its absence decreases ATP-coupled respiration in murine embryonic fibroblasts[46–49]. Because Mfn-2 has been implicated in LD-mitochondria interactions and FAO in brown adipocytes[50], we evaluated whether exercise modifies Mfn-2 levels in the murine NAFLD models. We found that exercise increased the content of Mfn-2 in liver lysates of HFD- and MCD-fed mice (Fig. 8A,B). In MCD-fed mice, this increase occurred mainly in the PDM (Supplementary Fig. 10A). To directly test the importance of Mfn-2 for LD-mitochondria contacts, we quantified these interactions in the hepatic tissues of Mfn-2 L-KO mice. As reported[30], the size of liver LDs is greater in Mfn-2 L-KO mice on a chow diet than in WT mice (Supplementary Fig. 10B,C). In contrast to what has been reported in brown adipocytes[50], the number of LD-mitochondria interactions in liver was higher in Mfn-2 L-KO mice than in WT mice (Supplementary Fig. 10D), indicating that Mfn-2 is not required for the formation of LD-mitochondria contacts in the liver.

To test the importance of Mfn-2 in the exercise-induced effects on LD-mitochondria interactions, WT and Mfn-2 L-KO mice were placed on HFD in an attempt to standardize the nutritional stress and to increase LD numbers and their interactions with mitochondria. Results showed that hepatic fibrosis was greater in Mfn-2 L-KO mice than in WT mice, and exercise had no effect on fibrosis levels in Mfn-2 L-KO mice (Fig. 8C). We next evaluated whether Mfn-2 is involved in the changes in hepatic FAO rates induced by exercise. Aerobic exercise increased FAO rates in hepatic PDM but not in CM in WT mice, but this effect was lost in Mfn-2 L-KO mice (Fig. 8D). This indicates that Mfn-2 is necessary for the elevated FAO induced by exercise exclusively in the mitochondria that are bound to LDs.

The composition of membrane phospholipids regulates mitochondrial membrane curvature, which is critical for mitochondrial structure and function[51]. Additionally, the

activity of carnitine palmitoyl transferase 1a (CPT1a), which catalyzes the transport of long-chain fatty acids into the mitochondria matrix for β -oxidation, is modulated by mitochondrial morphology [52]. It was recently reported that Mfn-2 can bind and extract phosphatidylserine from membranes to create rigid domains of phospholipids [30]. To question the mechanistic role of Mfn-2 in the exercise-induced changes in PDM, we evaluated its ability to bind phospholipids, and confirmed that Mfn-2 was able to bind phosphatidylserine, but with specific binding to phosphatidate and cardiolipin (Supplementary Fig. 11A). Of note, phosphatidate is a negatively charged phospholipid that can change membrane curvature [53].

Analysis of phosphatidate levels in the PDM revealed higher levels in HFD+Ex mice than in sedentary HFD mice (Supplementary Fig. 11B), suggesting that elevated phosphatidate in PDM (due to Mfn-2 aggregating phospholipids) might promote the negative curvature of the mitochondrial membrane, decreasing its circularity value. Circularity values close to zero indicate elongated mitochondria, whereas values close to 1 indicate a perfectly circular mitochondrion [22]. Exercise decreased the circularity in HFD and MCD-fed mice (Fig. 8E). Interestingly, Mfn-2 deficiency in the liver increased the circularity of mitochondria (Supplementary Fig. 10E). Additionally, exercise had no effect on the circularity of mitochondria in Mfn-2 L-KO mice (Fig. 8E). To gain insights into the effects of exercise and Mfn-2 in mitochondrial membrane lipid composition, we quantified the fatty acid composition of PDM (Supplementary Fig. 12). We found that exercise decreased saturated fatty acid levels and increased monounsaturated fatty acid levels when compared with sedentary WT mice. Importantly, this effect was lost in Mfn-2 L-KO mice, suggesting that Mfn-2 is required to remodel mitochondrial lipids in response to exercise. Concordantly, hepatic PDM of Mfn-2 L-KO mice had higher SFA levels and lower MUFA than WT mice (Supplementary Fig. 12).

DISCUSSION

The beneficial effects of exercise on NAFLD progression have been consistently reported in the literature [8]. However, it remains uncertain whether these effects are associated with **changes in** hepatic LD-mitochondria interactions. In the present study, we show that the number of LD-mitochondria contacts increases in both HFD- and MCD-induced

1 NAFLD murine models, independent of the LD number, and that the number of these
2 contacts directly correlates with insulin resistance and circulating transaminases. We also
3 show that a moderate-intensity aerobic exercise program decreases the number of LD-
4 mitochondria contacts and results in microvesicular hepatic steatosis associated with
5 improved insulin resistance and transaminase levels and higher hepatic FAO rates in
6 PDM. At the molecular level, exercise increased the content of Mfn-2 in PDM, and Mfn-
7 2 was required for increased FAO in PDM through changes in the mitochondrial
8 membrane curvature.
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10 As LD-mitochondria interactions were elevated in two different dietary models of
11 NAFLD (HFD and MCD), it is possible that the formation of these contacts is
12 nonspecifically dependent on LD expansion. Our observations are consistent with a study
13 that used HFD for 12 weeks [54] but not with another that fed mice with HFD for 9 weeks
14 [55]. Thus, it is possible that increased LD-mitochondria interactions are directly related
15 to the severity of NAFLD. To the best of our knowledge, no studies of LD-mitochondria
16 contacts have been reported in humans with NAFLD. However, studies in mice and
17 humans have shown that PLIN5 is more abundant in NAFLD [56]. Because PLIN5 is a
18 proposed protein tether for LD-mitochondria contacts, it is possible that increased PLIN5
19 levels in humans with NAFLD reflect a higher number of LD-mitochondria contacts in
20 hepatocytes.
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22 The impact of exercise on LD-mitochondria contacts in the liver is unknown. It is
23 important to determine whether these interactions regulate LD expansion or utilization in
24 hepatocytes to prevent or promote lipotoxicity. Our findings support the hypothesis that
25 these interactions promote LD expansion in NAFLD. Indeed, the positive correlation
26 between the LD-mitochondria contacts number and mitochondria-bound LD size
27 suggests that these interactions promote LD growth. This may also explain the positive
28 correlation between LD-mitochondria interactions and NAFLD score, as larger LDs are
29 linked to increased NAFLD severity [18]. Concordantly, we previously reported that
30 exposure to oleic acid increases LD-mitochondria interactions in HepG2 hepatocytes
31 [57]. Oleic acid exposure generates large LDs and LD expansion; thus, greater LD-
32 mitochondria contacts may mediate oleic acid-induced LD expansion. In addition, liver
33 PLIN5 overexpression exacerbates steatosis in HFD-fed mice [29].
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35 In the NAFLD models used here, mitochondria bound to LDs (PDM) showed a
36 greater ratio of ATP-coupled respiration and lower FAO than free CM, a feature
37 associated with greater LD expansion in BAT [21]. It is plausible that PDM locally
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1 generate ATP, which requires the activation of acyl-CoA synthetases for the esterification
2 of fatty acids into triglycerides as LDs expand [45]. In addition, PDM have a high capacity
3 to process pyruvate and thus increase the malonyl-CoA pool, ultimately promoting *de*
4 *novo* lipogenesis [58]. It has been reported that hepatic PDM but not CM are enriched in
5 **enzymes** involved in lipid anabolism, facilitating fatty acid esterification and LD
6 expansion under conditions of nutrient excess [59]. Mitochondria lack the necessary
7 enzymes for triglyceride synthesis, which are present in the endoplasmic reticulum. This
8 is concordant with the observation that PDM in hepatocytes [59] and adipocytes [60] are
9 in close contact with the endoplasmic reticulum and that PDM have distinctive features
10 and collaborate with the endoplasmic reticulum for LD expansion, a mechanism that we
11 cannot rule out as the PDM also contain endoplasmic reticulum fractions.

12 We found that MCD-induced NASH also increased LD-mitochondria contacts in
13 hepatocytes. Importantly, the MCD model induces LD accumulation in the liver by
14 increasing fatty acid uptake and inhibiting triglyceride export, without significantly
15 affecting FAO [61]. **Furthermore, we** found that the MCD-induced NASH model
16 generated larger LDs bound to mitochondria and more LD-mitochondria interactions than
17 the HFD-induced SHS model, (31.3% in MCD vs. 21.6% in HFD). Taken together, our
18 data suggests that LD-mitochondrial contacts have a general mechanism that allows LD
19 expansion in NAFLD.

20 An important finding of our study is that exercise decreases the number of hepatic
21 LD-mitochondria interactions in NAFLD models. Strikingly, exercise reduced the size of
22 LDs bound to mitochondria in the NAFLD models, but it did not affect the size of LDs
23 that were not bound to mitochondria. The differential effect of exercise on LDs
24 subpopulations has been described in muscle [22], although the mechanisms regulating
25 such selectivity remain unknown.

26 We found that exercise increased the elongated morphology **of mitochondria**,
27 ATP-coupled respiration, and FAO of PDM in the SHS model. **The smaller size of LDs**
28 **bound to mitochondria after exercise may be the result of increased lipolysis of LD's**
29 **triglycerides and increased FAO.** Considering that hepatic PDM functionally promote
30 LD expansion under normal and NAFLD conditions, our results **support the idea** that
31 exercise modifies hepatic PDM function to increase LD utilization in NAFLD. Notably,
32 exercise-induced increase in FAO was only observed in PDM, but not in CM, which
33 indeed showed a reduction in FAO after exercise. This selective effect on PDM
34 functionality seems plausible, as it has been shown that PLIN5 promotes LD-

mitochondria interactions and LD expansion in the liver under basal and HFD conditions [26,29,56]. Contrastingly, PKA phosphorylation of PLIN5 at serine¹⁵⁵ in hepatocytes enhances lipolysis of LDs [62]. Additionally, starved cells have increased the transfer of fatty acids from LDs to mitochondria for oxidation [27,63]. Importantly, the change in the functionality of hepatic PDM induced by exercise, switching from a "lipogenic" to a "FAO" phenotype, boosts FAO upon increased energy requirement conditions and tempers, at the same time, energy demanding process such as lipogenesis. Consequently, exercise determines a transition of hepatic LD-mitochondria functionality from LD expansion to LD utilization.

We found that Mfn-2 deficiency was associated with increased LD-mitochondria contacts and decreased FAO in the liver. Intriguingly, lower Mfn-2 levels in PDM increased LD-mitochondria interactions in the NAFLD model while decreasing FAO. Mfn-2 levels are associated with NAFLD severity. Mfn-2 levels are decreased in patients with NASH and Mfn-2 ablation in mice causes a NASH-like phenotype that promotes hepatocarcinoma [30]. Here, we show that the beneficial effects of exercise in NAFLD are associated with a reduced number of LD-mitochondria interactions and increased FAO in PDM, likely due to Mfn-2 enrichment in PDM.

Our results suggest that Mfn-2 regulates membrane curvature to promote FAO in PDM through its interaction and capacity to form specific domains with membrane phospholipids. We found that Mfn-2 binds to phosphatidate, and this mechanism may promote a negative curvature of the mitochondrial membranes, resulting in a decreased circularity value. Indeed, Mfn-2 promotes changes in the curvature of PDM, favoring CPT1a activity [52] enhancing FAO. In addition, we found an increase in the relative content of saturated fatty acids in the hepatic mitochondrial membranes of exercised animals, suggesting that membrane fluidity is decreased in PDM, while no changes in FAO or in saturated fatty acids were observed in exercised Mfn2 KO mice, indicating that Mfn2 participates in the regulation of mitochondrial membranes fatty acid composition in response to exercise. While a specific analysis of PDM and CM mitochondrial subpopulations has not been conducted, previous findings indicate that mitochondrial morphology plays a role in regulating FAO in hepatocytes [52].

Larger LDs have been shown to be associated to NAFLD progression and increased stellate cell activation, inflammation, and stress [43]. Therefore, the microvesicular steatosis pattern that we found in mice subjected to aerobic exercise may be one mechanism by which exercise reduces NAFLD progression. Exercise likely

improves NALFD severity by reducing the number of LD-mitochondria contacts and, consequently, the LD size. In addition, small LDs increase the ratio of LD surface area to LD volume, possibly boosting lipolysis efficiency by providing greater access to lipases [22].

A relevant consideration in the analysis of the results is the difference in body weight between exercised and sedentary mice on HFD. Several of the effects of aerobic exercise on key metabolic variables such as glucose tolerance, cholesterol, and transaminase levels may be in part related to changes in body weight. Thus, ANCOVA analysis with body weight as a covariant was performed, following recent guidelines recommendations [64–66]. These analyses indicated that no significant differences existed between exercised and sedentary animals in NAFLD score, glucose tolerance and transaminase levels once the relationship with body weight was included in the modeling. Nevertheless, the effects of exercise on HOMA-IR and liver triglycerides remained independent of changes in body weight.

Among the limitations of our study is that other types of exercise training (e.g., resistance training) were not performed, thus our conclusions are strictly restricted to continuous moderate-intensity aerobic exercise. Also, as this study was focused on the effect of exercise on the liver, only the weight of the gastrocnemius and soleus of the left leg, as well as the eWAT, were measured. Thus, the lack of quantification of fat or lean body mass prevented us from determining the effects of exercise on body composition. Finally, only male mice were included in this study, so further research is necessary to validate these results in female animals. However, despite these caveats, our study is strengthened by the high consistency of the findings across independent animal cohorts, increasing our confidence in the reproducibility of our results.

From a translational perspective, our findings that exercise-induced energy demands determine regulated changes in the physical and functional relationships between LDs and mitochondria, resulting in increased FAO in PDM and decreased severity of NAFLD, may drive the investigation of the molecular mediators and mechanisms of these adaptations that may result in novel strategies to prevent NAFLD progression. Considering the roles of Mfn-2 in mitochondrial morphology and function in the liver, therapeutic manipulations of Mfn2 levels/activity may ameliorate inflammation and fibrosis in NAFLD.

In conclusion, herein we show in two different murine models of NAFLD that aerobic exercise decreases NAFLD severity by reducing the interactions between hepatic

LDs and mitochondria, as well as by decreasing LD size. Additionally, we found that aerobic exercise increases FAO in PDM and this process depends on Mfn-2 enrichment, which modifies lipid droplet-mitochondria communication in the liver of mice.

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Credit authorship contribution statement

Juan Carlos Borquez conceived the study, performed experiments, analyzed the data and wrote the manuscript. **Francisco Díaz-Castro** performed experiments. **Francisco Pino-de la Fuente** performed experiments. **Karla Espinoza** performed experiments. **Ana María Figueroa** performed experiments. **Inma Martínez-Ruiz** performed experiments. **Vanessa Hernández** performed experiments. **Iliana López-Salgado** performed experiments. **Raúl Ventura** performed experiments. **Joan Carles Domingo** performed experiments and analyzed the data. **Marta Bosch** analyzed the data. **Alba Fajardo** performed experiments. **David Sebastian** performed experiments and wrote the manuscript. **Alejandra Espinoza** analyzed the data. **Albert Pol** analyzed the data. **Antonio Zorzano** analyzed the data and wrote the manuscript. **Víctor Cortés** conceived the study, analyzed the data, wrote the manuscript, and approved the final version of the manuscript for publication. **María Isabel Hernández-Alvarez** conceived the study, analyzed the data, wrote the manuscript, and approved the final version of the manuscript for publication. **Rodrigo Troncoso** conceived the study, analyzed the data, wrote the manuscript, and approved the final version of the manuscript for publication. All the authors contributed to editing.

REFERENCES

[1] Riazi K, Azhari H, Charette JH, Underwood FE, King JA, Afshar EE, et al. The prevalence and incidence of NAFLD worldwide: a systematic review and meta-analysis.

Lancet Gastroenterol Hepatol 2022;7:851–61. [https://doi.org/10.1016/S2468-1253\(22\)00165-0](https://doi.org/10.1016/S2468-1253(22)00165-0).

[2] Targher G, Byrne CD, Tilg H. NAFLD and increased risk of cardiovascular disease: clinical associations, pathophysiological mechanisms and pharmacological implications. *Gut* 2020;69:1691–705. <https://doi.org/10.1136/gutjnl-2020-320622>.

[3] Golabi P, Paik JM, Kumar A, Al Shabeeb R, Eberly KE, Cusi K, et al. Nonalcoholic fatty liver disease (NAFLD) and associated mortality in individuals with type 2 diabetes, pre-diabetes, metabolically unhealthy, and metabolically healthy individuals in the United States. *Metabolism* 2023;146:155642. <https://doi.org/10.1016/j.metabol.2023.155642>.

[4] Guarino M, Kumar P, Felser A, Terracciano LM, Guixé-Muntet S, Humar B, et al. Exercise Attenuates the Transition from Fatty Liver to Steatohepatitis and Reduces Tumor Formation in Mice. *Cancers* 2020;12:1407. <https://doi.org/10.3390/cancers12061407>.

[5] Alex S, Boss A, Heerschap A, Kersten S. Exercise training improves liver steatosis in mice. *Nutr Metab* 2015;12:29. <https://doi.org/10.1186/s12986-015-0026-1>.

[6] Fredrickson G, Barrow F, Dietsche K, Parthiban P, Khan S, Robert S, et al. Exercise of high intensity ameliorates hepatic inflammation and the progression of NASH. *Mol Metab* 2021;53:101270. <https://doi.org/10.1016/j.molmet.2021.101270>.

[7] Linden MA, Sheldon RD, Meers GM, Ortinau LC, Morris EM, Booth FW, et al. Aerobic exercise training in the treatment of non-alcoholic fatty liver disease related fibrosis. *J Physiol* 2016;594:5271–84. <https://doi.org/10.1113/JP272235>.

[8] Pino-de la Fuente F, Bórquez JC, Díaz-Castro F, Espinosa A, Chiong M, Troncoso R. Exercise regulation of hepatic lipid droplet metabolism. *Life Sci* 2022;298:120522. <https://doi.org/10.1016/j.lfs.2022.120522>.

[9] Brouwers B, Hesselink MKC, Schrauwen P, Schrauwen-Hinderling VB. Effects of exercise training on intrahepatic lipid content in humans. *Diabetologia* 2016;59:2068–79. <https://doi.org/10.1007/s00125-016-4037-x>.

[10] Brouwers B, Schrauwen-Hinderling VB, Jelenik T, Gemmink A, Sparks LM, Havekes B, et al. Exercise training reduces intrahepatic lipid content in people with and people without nonalcoholic fatty liver. *Am J Physiol Endocrinol Metab* 2018;314:E165–73. <https://doi.org/10.1152/ajpendo.00266.2017>.

[11] Marcinko K, Sikkema SR, Samaan MC, Kemp BE, Fullerton MD, Steinberg GR. High intensity interval training improves liver and adipose tissue insulin sensitivity. *Mol Metab* 2015;4:903–15. <https://doi.org/10.1016/j.molmet.2015.09.006>.

[12] la Fuente FP, Quezada L, Sepúlveda C, Monsalves-Alvarez M, Rodríguez JM, Sacristán C, et al. Exercise regulates lipid droplet dynamics in normal and fatty liver. *Biochim Biophys Acta BBA - Mol Cell Biol Lipids* 2019;1864:158519. <https://doi.org/10.1016/j.bbalip.2019.158519>.

[13] Winn NC, Liu Y, Rector RS, Parks EJ, Ibdah JA, Kanaley JA. Energy-matched moderate and high intensity exercise training improves nonalcoholic fatty liver disease risk independent of changes in body mass or abdominal adiposity - A randomized trial. *Metabolism* 2018;78:128–40. <https://doi.org/10.1016/j.metabol.2017.08.012>.

[14] Ok D-P, Ko K, Bae JY. Exercise without dietary changes alleviates nonalcoholic fatty liver disease without weight loss benefits. *Lipids Health Dis* 2018;17:207. <https://doi.org/10.1186/s12944-018-0852-z>.

[15] Harris SJ, Smith N, Hummer B, Schreiberman IR, Faust AJ, Geyer NR, et al. Exercise training improves serum biomarkers of liver fibroinflammation in patients with metabolic dysfunction-associated steatohepatitis. *Liver Int Off J Int Assoc Study Liver* 2023. <https://doi.org/10.1111/liv.15769>.

- [16] Gluchowski NL, Becuwe M, Walther TC, Farese RV. Lipid droplets and liver disease: from basic biology to clinical implications. *Nat Rev Gastroenterol Hepatol* 2017;14:343–55. <https://doi.org/10.1038/nrgastro.2017.32>.
- [17] Olzmann JA, Carvalho P. Dynamics and functions of lipid droplets. *Nat Rev Mol Cell Biol* 2019;20:137–55. <https://doi.org/10.1038/s41580-018-0085-z>.
- [18] Mashek DG. Hepatic lipid droplets: A balancing act between energy storage and metabolic dysfunction in NAFLD. *Mol Metab* 2021;50:101115. <https://doi.org/10.1016/j.molmet.2020.101115>.
- [19] Valm AM, Cohen S, Legant WR, Melunis J, Hershberg U, Wait E, et al. Applying systems-level spectral imaging and analysis to reveal the organelle interactome. *Nature* 2017;546:162–7. <https://doi.org/10.1038/nature22369>.
- [20] Cui L, Mirza AH, Zhang S, Liang B, Liu P. Lipid droplets and mitochondria are anchored during brown adipocyte differentiation. *Protein Cell* 2019;10:921–6. <https://doi.org/10.1007/s13238-019-00661-1>.
- [21] Benador IY, Veliova M, Mahdavian K, Petcherski A, Wikstrom JD, Assali EA, et al. Mitochondria Bound to Lipid Droplets Have Unique Bioenergetics, Composition, and Dynamics that Support Lipid Droplet Expansion. *Cell Metab* 2018;27:869–885.e6. <https://doi.org/10.1016/j.cmet.2018.03.003>.
- [22] de Almeida ME, Nielsen J, Petersen MH, Wentorf EK, Pedersen NB, Jensen K, et al. Altered intramuscular network of lipid droplets and mitochondria in type 2 diabetes. *Am J Physiol Cell Physiol* 2023;324:C39–57. <https://doi.org/10.1152/ajpcell.00470.2022>.
- [23] Tarnopolsky MA, Rennie CD, Robertshaw HA, Fedak-Tarnopolsky SN, Devries MC, Hamadeh MJ. Influence of endurance exercise training and sex on intramyocellular lipid and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. *Am J Physiol Regul Integr Comp Physiol* 2007;292:R1271–1278. <https://doi.org/10.1152/ajpregu.00472.2006>.
- [24] Najt CP, Adhikari S, Heden TD, Cui W, Gansemer ER, Rauckhorst AJ, et al. Organelle interactions compartmentalize hepatic fatty acid trafficking and metabolism. *Cell Rep* 2023;42:112435. <https://doi.org/10.1016/j.celrep.2023.112435>.
- [25] Talari NK, Mattam U, Meher NK, Paripati AK, Mahadev K, Krishnamoorthy T, et al. Lipid-droplet associated mitochondria promote fatty-acid oxidation through a distinct bioenergetic pattern in male Wistar rats. *Nat Commun* 2023;14:766. <https://doi.org/10.1038/s41467-023-36432-0>.
- [26] Wang H, Sreenivasan U, Hu H, Saladino A, Polster BM, Lund LM, et al. Perilipin 5, a lipid droplet-associated protein, provides physical and metabolic linkage to mitochondria. *J Lipid Res* 2011;52:2159–68. <https://doi.org/10.1194/jlr.M017939>.
- [27] Miner GE, So CM, Edwards W, Herring LE, Coleman RA, Klett EL, et al. Perilipin 5 interacts with Fatp4 at membrane contact sites to promote lipid droplet-to-mitochondria fatty acid transport 2022:2022.02.03.479028. <https://doi.org/10.1101/2022.02.03.479028>.
- [28] Keenan SN, Meex RC, Lo JCY, Ryan A, Nie S, Montgomery MK, et al. Perilipin 5 Deletion in Hepatocytes Remodels Lipid Metabolism and Causes Hepatic Insulin Resistance in Mice. *Diabetes* 2019;68:543–55. <https://doi.org/10.2337/db18-0670>.
- [29] Trevino MB, Mazur-Hart D, Machida Y, King T, Nadler J, Galkina EV, et al. Liver Perilipin 5 Expression Worsens Hepatosteatosis But Not Insulin Resistance in High Fat-Fed Mice. *Mol Endocrinol Baltim Md* 2015;29:1414–25. <https://doi.org/10.1210/me.2015-1069>.
- [30] Hernández-Alvarez MI, Sebastián D, Vives S, Ivanova S, Bartoccioni P,

Kakimoto P, et al. Deficient Endoplasmic Reticulum-Mitochondrial Phosphatidylserine Transfer Causes Liver Disease. *Cell* 2019;177:881-895.e17.

<https://doi.org/10.1016/j.cell.2019.04.010>.

[31] Sebastián D, Hernández-Alvarez MI, Segalés J, Sorianello E, Muñoz JP, Sala D, et al. Mitofusin 2 (Mfn2) links mitochondrial and endoplasmic reticulum function with insulin signaling and is essential for normal glucose homeostasis. *Proc Natl Acad Sci* 2012;109:5523–8. <https://doi.org/10.1073/pnas.1108220109>.

[32] Mfn2 is critical for brown adipose tissue thermogenic function. *EMBO J* 2017;36:1543–58. <https://doi.org/10.15252/embj.201694914>.

[33] Chen H, McCaffery JM, Chan DC. Mitochondrial Fusion Protects against Neurodegeneration in the Cerebellum. *Cell* 2007;130:548–62. <https://doi.org/10.1016/j.cell.2007.06.026>.

[34] Liang W, Menke AL, Driessen A, Koek GH, Lindeman JH, Stoop R, et al. Establishment of a general NAFLD scoring system for rodent models and comparison to human liver pathology. *PloS One* 2014;9:e115922. <https://doi.org/10.1371/journal.pone.0115922>.

[35] Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: Open source software for digital pathology image analysis. *Sci Rep* 2017;7:16878. <https://doi.org/10.1038/s41598-017-17204-5>.

[36] Tapia PJ, Figueroa A-M, Eisner V, González-Hódar L, Robledo F, Agarwal AK, et al. Absence of AGPAT2 impairs brown adipogenesis, increases IFN stimulated gene expression and alters mitochondrial morphology. *Metabolism* 2020;111:154341. <https://doi.org/10.1016/j.metabol.2020.154341>.

[37] Acín-Perez R, Petcherski A, Veliova M, Benador IY, Assali EA, Colletuori G, et al. Recruitment and remodeling of peridroplet mitochondria in human adipose tissue. *Redox Biol* 2021;46:102087. <https://doi.org/10.1016/j.redox.2021.102087>.

[38] Bosch M, Sánchez-Álvarez M, Fajardo A, Kapetanovic R, Steiner B, Dutra F, et al. Mammalian lipid droplets are innate immune hubs integrating cell metabolism and host defense. *Science* 2020;370:eaay8085. <https://doi.org/10.1126/science.aay8085>.

[39] Ngo J, Benador IY, Brownstein AJ, Vergnes L, Veliova M, Shum M, et al. Isolation and functional analysis of peridroplet mitochondria from murine brown adipose tissue. *STAR Protoc* 2021;2:100243. <https://doi.org/10.1016/j.xpro.2020.100243>.

[40] Sebastián D, Herrero L, Serra D, Asins G, Hegardt FG. CPT I overexpression protects L6E9 muscle cells from fatty acid-induced insulin resistance. *Am J Physiol Endocrinol Metab* 2007;292:E677-686. <https://doi.org/10.1152/ajpendo.00360.2006>.

[41] Castillo Salinas F, Montaner Ramón A, Castillo Ferrer F-J, Domingo-Carnice A, Cordobilla B, Domingo JC. Erythrocyte Membrane Docosahexaenoic Acid (DHA) and Lipid Profile in Preterm Infants at Birth and Over the First Month of Life: A Comparative Study with Infants at Term. *Nutrients* 2022;14:4956. <https://doi.org/10.3390/nu14234956>.

[42] Lord SJ, Velle KB, Mullins RD, Fritz-Laylin LK. SuperPlots: Communicating reproducibility and variability in cell biology. *J Cell Biol* 2020;219:e202001064. <https://doi.org/10.1083/jcb.202001064>.

[43] Scorletti E, Carr RM. A new perspective on NAFLD: Focusing on lipid droplets. *J Hepatol* 2021:S0168827821021814. <https://doi.org/10.1016/j.jhep.2021.11.009>.

[44] Shum M, Ngo J, Shirihai OS, Liesa M. Mitochondrial oxidative function in NAFLD: Friend or foe? *Mol Metab* 2021;50:101134. <https://doi.org/10.1016/j.molmet.2020.101134>.

[45] Benador IY, Veliova M, Liesa M, Shirihai OS. Mitochondria Bound to Lipid

Droplets: Where Mitochondrial Dynamics Regulate Lipid Storage and Utilization. *Cell Metab* 2019;29:827–35. <https://doi.org/10.1016/j.cmet.2019.02.011>.

[46] Hu Y, Chen H, Zhang L, Lin X, Li X, Zhuang H, et al. The AMPK-MFN2 axis regulates MAM dynamics and autophagy induced by energy stresses. *Autophagy* 2021;17:1142–56. <https://doi.org/10.1080/15548627.2020.1749490>.

[47] Bach D, Pich S, Soriano FX, Vega N, Baumgartner B, Oriola J, et al. Mitofusin-2 Determines Mitochondrial Network Architecture and Mitochondrial Metabolism. *J Biol Chem* 2003;278:17190–7. <https://doi.org/10.1074/jbc.M212754200>.

[48] Muñoz JP, Ivanova S, Sánchez-Wandelmer J, Martínez-Cristóbal P, Noguera E, Sancho A, et al. Mfn2 modulates the UPR and mitochondrial function via repression of PERK. *EMBO J* 2013;32:2348–61. <https://doi.org/10.1038/emboj.2013.168>.

[49] Sebastián D, Hernández-Alvarez MI, Segalés J, Sorianello E, Muñoz JP, Sala D, et al. Mitofusin 2 (Mfn2) links mitochondrial and endoplasmic reticulum function with insulin signaling and is essential for normal glucose homeostasis. *Proc Natl Acad Sci* 2012;109:5523–8. <https://doi.org/10.1073/pnas.1108220109>.

[50] Boutant M, Kulkarni SS, Joffraud M, Ratajczak J, Valera-Alberni M, Combe R, et al. Mfn2 is critical for brown adipose tissue thermogenic function. *EMBO J* 2017;36:1543–58. <https://doi.org/10.15252/embj.201694914>.

[51] Dong J, Ye F, Lin J, He H, Song Z. The metabolism and function of phospholipids in Mitochondria. *Mitochondrial Commun* 2023;1:2–12. <https://doi.org/10.1016/j.mitoco.2022.10.002>.

[52] Mitochondrial morphology controls fatty acid utilization by changing CPT1 sensitivity to malonyl- CoA n.d. <https://doi.org/10.15252/embj.2022111901>.

[53] Phosphatidic acid in membrane rearrangements - Zhukovsky - 2019 - FEBS Letters - Wiley Online Library n.d. <https://febs.onlinelibrary.wiley.com/doi/10.1002/1873-3468.13563> (accessed May 19, 2023).

[54] Krahmer N, Najafi B, Schueder F, Quagliarini F, Steger M, Seitz S, et al. Organellar Proteomics and Phospho-Proteomics Reveal Subcellular Reorganization in Diet-Induced Hepatic Steatosis. *Dev Cell* 2018;47:205–221.e7. <https://doi.org/10.1016/j.devcel.2018.09.017>.

[55] Khan SA, Wollaston-Hayden EE, Markowski TW, Higgins L, Mashek DG. Quantitative analysis of the murine lipid droplet-associated proteome during diet-induced hepatic steatosis. *J Lipid Res* 2015;56:2260–72. <https://doi.org/10.1194/jlr.M056812>.

[56] Wang C, Zhao Y, Gao X, Li L, Yuan Y, Liu F, et al. Perilipin 5 improves hepatic lipotoxicity by inhibiting lipolysis. *Hepatol Baltim Md* 2015;61:870–82. <https://doi.org/10.1002/hep.27409>.

[57] Eynaudi A, Díaz-Castro F, Bórquez JC, Bravo-Sagua R, Parra V, Troncoso R. Differential Effects of Oleic and Palmitic Acids on Lipid Droplet-Mitochondria Interaction in the Hepatic Cell Line HepG2. *Front Nutr* 2021;8.

[58] Benador IY, Veliova M, Liesa M, Shirihai OS. Mitochondria Bound to Lipid Droplets: Where Mitochondrial Dynamics Regulate Lipid Storage and Utilization. *Cell Metab* 2019;29:827–35. <https://doi.org/10.1016/j.cmet.2019.02.011>.

[59] Najt CP, Adhikari S, Heden TD, Cui W, Gansemer ER, Rauckhorst AJ, et al. Organelle interactions compartmentalize hepatic fatty acid trafficking and metabolism. *Cell Rep* 2023;42:112435. <https://doi.org/10.1016/j.celrep.2023.112435>.

[60] Freyre CAC, Rauher PC, Ejsing CS, Klemm RW. MIGA2 Links Mitochondria, the ER, and Lipid Droplets and Promotes De Novo Lipogenesis in Adipocytes. *Mol Cell* 2019;76:811–825.e14. <https://doi.org/10.1016/j.molcel.2019.09.011>.

- [61] Rinella ME, Elias MS, Smolak RR, Fu T, Borensztajn J, Green RM. Mechanisms of hepatic steatosis in mice fed a lipogenic methionine choline-deficient diet. *J Lipid Res* 2008;49:1068–76. <https://doi.org/10.1194/jlr.M800042-JLR200>.
- [62] Keenan SN, De Nardo W, Lou J, Schittenhelm RB, Montgomery MK, Granneman JG, et al. Perilipin 5 S155 phosphorylation by PKA is required for the control of hepatic lipid metabolism and glycemic control. *J Lipid Res* 2021;62:100016. <https://doi.org/10.1194/jlr.RA120001126>.
- [63] Rambold AS, Cohen S, Lippincott-Schwartz J. Fatty acid trafficking in starved cells: regulation by lipid droplet lipolysis, autophagy, and mitochondrial fusion dynamics. *Dev Cell* 2015;32:678–92. <https://doi.org/10.1016/j.devcel.2015.01.029>.
- [64] Mina AI, LeClair RA, LeClair KB, Cohen DE, Lantier L, Banks AS. CalR: A Web-Based Analysis Tool for Indirect Calorimetry Experiments. *Cell Metab* 2018;28:656–666.e1. <https://doi.org/10.1016/j.cmet.2018.06.019>.
- [65] Tschöp MH, Speakman JR, Arch JRS, Auwerx J, Brüning JC, Chan L, et al. A guide to analysis of mouse energy metabolism. *Nat Methods* 2011;9:57–63. <https://doi.org/10.1038/nmeth.1806>.
- [66] Fernández-Verdejo R, Ravussin E, Speakman JR, Galgani JE. Progress and challenges in analyzing rodent energy expenditure. *Nat Methods* 2019;16:797–9. <https://doi.org/10.1038/s41592-019-0513-9>.

FIGURE LEGENDS

Figure 1. Aerobic exercise decreases disease severity and improves exercise capacity in mice with HFD-induced SHS. **A)** Maximal speed. **B)** Glucose tolerance test (GTT) and area under the curve (AUC) of GTT. **C)** Fasting glycemia. **D)** Total cholesterol. **E)** Serum triglycerides. **F)** Alanine aminotransferase and aspartate transaminase. Data presented as mean $\pm$ SEM. n=9-13 mice per group. 2-way ANOVA followed by Tukey's posterior test for multiple comparisons (in case of interaction between variables) were performed. Statistical significance $p < 0.05$; *Differences between groups indicated, *Diet *Exercise. CD: control diet; CD+Ex: control diet + exercise; HFD: high-fat diet; HFD+Ex: high-fat diet + exercise.

Figure 2. Aerobic exercise reduces hepatic lipid droplet size without altering hepatic mitochondrial morphology in mice with HFD-induced SHS. **A)** Liver (H&E stain histology, 20 $\times$). **B)** Lipid droplet number. **C)** Lipid droplet size frequency. **D)** NAFLD score. **E)** liver triglycerides. Linear regression using body weight gain as a covariate. **(F)** HOMA-IR, **G)** liver triglycerides, and **H)** fasting glycemia. **I)** Representative micrographs of liver (8200 $\times$, scale bar 2 μ m). Blue arrow: Mitochondria. Yellow arrow: Lipid droplet. **J)** Mitochondria number, size, aspect ratio, and density from electron microscopy analyses. n=4 per group, each point is an average (color points) and an individual micrograph (white points) analyzed by TEM. Data presented as mean $\pm$ SEM. n=9-13 mice per group. 2-way ANOVA and ANCOVA were performed. Statistical significance $p < 0.05$; *Diet, *Exercise. CD: control diet; CD+Ex: control diet + exercise; HFD: high-fat diet; HFD+Ex: high-fat diet + exercise.

Figure 3. Aerobic exercise limits lipid droplet-mitochondria interactions in hepatocytes and is associated with lower ALT transaminase levels and area under the curve of glucose tolerance test in mice with HFD-induced SHS. **A)** Representative micrograph of murine liver (large micrograph; 8200 $\times$, scale bar 1 μ m. small micrograph; 28000 $\times$, scale bar 500 nm) and **B)** LD-mitochondria interactions (physical contact between the edge of the LD surface and the outer membrane of the mitochondrion by electron microscopy was considered LD-mitochondria interaction) normalized to the total number of mitochondria. **C)** LD-mitochondria interactions normalized to the total number of LDs. Correlation between LD-mitochondria interaction/ total number of mitochondria (%) and **D)** NAFLD score and **E)** ALT transaminase. **F)** Representative micrograph of murine liver (8200 $\times$, scale bar 1 μ m). **G)** Size of LDs bound to mitochondria (LD-M) and non-bound to mitochondria (LD-C). **H)** Correlation between size of LD-M and LD-mitochondria interaction/total number of mitochondria (%). Data presented as mean $\pm$ SEM. n=4 per group, each point is an average (color points) and an individual micrograph (white points) analyzed by TEM. In correlation graphs, each point is one mouse. 2-way ANOVA followed by Tukey's post-test (in case of interaction between variables) and Pearson's correlation coefficients were performed. Statistical significance $p < 0.05$; *Differences between groups. CD: control diet; CD+Ex: control diet + exercise; HFD: high-fat diet; HFD+Ex: high-fat diet + exercise; LD-M: Lipid droplet bound to mitochondria; LD-C: Lipid droplet non-bound to mitochondria.

Figure 4. Aerobic exercise increases ATP-coupled respiration of peridroplet mitochondria in mice with HFD-induced SHS. **A)** Representative micrograph of murine liver (12000 $\times$, scale bar 2 μ m). **B)** Mitochondria number. **C)** Mitochondria aspect ratio. **D)** Representative western blot images. **E)** Mitochondrial complex V, complex IV,

complex III, complex II, and complex I. **F)** Oxygen consumption with 5 mM glutamate/2.5 mM malate (baseline condition), 300 μ M adenosine diphosphate-ADP (ATP-coupled), and 1 μ M oligomycin (non-ATP-associated). Data presented as mean $\pm$ SEM. n=4 per group (TEM analyses). n=5-7 per group (western blotting and respirometry). 2-way ANOVA followed by Tukey's posterior test for multiple comparisons (in case of interaction between variables) were performed. Statistical significance $p < 0.05$; *Differences between groups indicated, *mitochondrial subpopulations *Exercise *Condition (Baseline and ATP-coupled). CM: cytoplasmic mitochondria; PDM: peridroplet mitochondria.

Figure 5. Hepatic LD-mitochondria interactions are increased in MCD-induced NASH. **A)** Protocol design. **B)** Body weight. **C)** Representative images of murine liver (H&E and Sirius Red stain, 20 $\times$, scale bar 100 μ m). **D)** Lipid droplet number. **E)** Lipid droplet size. **F)** NAFLD score. **G)** Sirius Red staining. **H)** Representative micrograph of murine liver (12000 $\times$, scale bar 2 μ m). **I)** LD-mitochondria interactions normalized to the total number of mitochondria. **J)** LD-mitochondria interactions normalized to the total number of lipid droplets. **K)** Mitochondria number, size, aspect ratio, and density from electron microscopy analyses. n=4-5 per group, each point is an average (color points) and an individual micrograph (white points) analyzed by TEM. Data presented as mean $\pm$ SEM. n=5 per group. Student's t-tests were performed. Statistical significance $p < 0.05$ *versus CD. CD: Control diet; MCD: methionine-choline deficient diet.

Figure 6. Aerobic exercise decreases liver collagen deposition and hepatic lipid droplet size and increases total mitochondria in MCD induced-NASH. **A)** Protocol design. **B)** Representative images of murine liver (H&E and Sirius Red histology, 20 $\times$, scale bar 100 μ m) **C)** NAFLD score. **D)** number of inflammatory foci. **E)** Sirius red staining. **F)** Lipid droplet number. **G)** Lipid droplet size. **H)** Liver triglycerides. **I)** Representative micrograph of murine liver (12000 $\times$, scale bar 2 μ m) and **J)** Mitochondria number, size, aspect ratio, and density from electron microscopy analyses. Each point is an average (color points) and an individual micrograph (white points) analyzed by TEM. Data presented as mean $\pm$ SEM. n=4-8 per group. Student's t-test was performed. Statistical significance $p < 0.05$ *versus MCD. MCD: MCD: methionine-choline deficient diet; MCD+Ex: methionine-choline deficient diet + exercise. LD-M: Lipid droplet bound to mitochondria. LD-C: Lipid droplet non-bound to mitochondria.

Figure 7. Aerobic exercise decreases hepatic LD-mitochondria interactions and the size of LDs bound to mitochondria in MCD-induced NASH. **A)** Representative micrographs of murine liver (25000 $\times$, scale bar 1 μ m). **B)** LD-mitochondria interactions normalized to the total number of mitochondria (%). **C)** LD-mitochondria interactions normalized to the total number of LDs. **D)** Size of LDs bound to mitochondria (LD-M) and non-bound to mitochondria (LD-C). **E)** Correlation between LD-M size and LD-mitochondria interaction/total number of mitochondria (%). **F)** Mitochondria aspect ratio of CM and PDM. Data presented as mean $\pm$ SEM. N=6-8 per group. Student's t-test, 2-way ANOVA followed by Tukey's post-test (in case of interaction between variables), and Pearson's correlation coefficient were performed. Statistical significance $p < 0.05$; *Differences between groups indicated, *mitochondrial subpopulations *Exercise. MCD: MCD: methionine-choline deficient diet; MCD+Ex: methionine-choline deficient diet+exercise; CM: cytoplasmic mitochondria; PDM: peridroplet mitochondria.

Figure 8. Aerobic exercise increases fatty acid oxidation in peridroplet mitochondria via Mitofusin-2 (Mfn-2) in HFD-induced SHS. **A)** Mfn-2 levels in whole cell lysates in HFD-induced SHS. **B)** Mfn-2 levels in whole cell lysates in MCD-induced NASH. **C)** Protocol design and Sirius Red analysis. **D)** Fatty acid oxidation assayed by labeled ¹⁴C-Oleic acid in PDM and CM. **E)** Circularity of PDM in SHS, NASH and Mfn-2 L-KO models. **F)** Proposed model. WT (n=6 mice) and Mfn2 L-KO (n=6-7 mice). Data presented as mean ± SEM. Student's t-test and 2-way ANOVA followed by Tukey's post-test were performed. Statistical significance p<0.05; *among groups, *Exercise *L-KO.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Isolation of lipid droplets. **A)** Protocol design. **B)** Representative images of the protocol phases. **C)** Purity of lipid droplet isolation by western blotting.

Supplementary Figure 2. Isolation of mitochondria subpopulation. **A)** Protocol design. **B)** Representative images of the protocol phases. **C)** Respiratory control ratio in oxygen consumption measures. **D)** and **E)** Purity of mitochondria subpopulation isolation by western blotting.

Supplementary Figure 3. Exercise decreases body weight and insulin resistance in SHS. **A)** Body weight. **B)** Body weight gain (8-12 weeks) **C)** Body weight gain (0-12 weeks) **D)** Caloric Intake. **E)** Grip strength. **F)** Serum insulin. **G)** Homeostatic Model Assessment of Insulin Resistance (HOMA-IR). **H)** LDL cholesterol. **I)** HDL cholesterol. Data presented as mean ± SEM. n=9-13 mice per group. ANCOVA and 2-way ANOVA were performed. Statistical significance p<0.05; *Diet, *Exercise. CD: control diet; CD+Ex: control diet + exercise; HFD: high-fat diet; HFD+Ex: high-fat diet + exercise.

Supplementary Figure 4. Exercise increases muscle fiber size and reduces adipocyte size in SHS. **A)** epididymal white adipose tissue weight (eWAT). **B)** eWAT (H&E stain histology, 20×). **C)** Average adipocyte size (eWAT). **D)** Gastrocnemius and Soleus muscle weight. **E)** Skeletal muscle (H&E stain histology, 20×). **F)** Average fiber size (gastrocnemius). Data presented as mean ± SEM. n=9-13 mice per group. 2-way ANOVA followed by Tukey's post-test. Statistical significance p<0.05; *Diet. *Interaction (* among indicated groups). CD: control diet; CD+Ex: control diet + exercise; HFD: high-fat diet; HFD+Ex: high-fat diet + exercise.

Supplementary Figure 5. Exercise reduces hepatic LD size in HFD-induced SHS. Representative images of murine liver and liver weight. Data presented as mean ± SEM. n=9-13 mice per group. 2-way ANOVA followed by Tukey's post-test. Statistical significance p<0.05; Interaction (*among indicated groups). CD: control diet; CD+Ex: control diet + exercise; HFD: high-fat diet; HFD+Ex: high-fat diet + exercise.

Supplementary Figure 6. Effect of body weight on exercise effects. Linear regression analysis using body weight gain as a covariate. **A)** NAFLD score. **B)** Area under the curve (AUC) of glucose tolerance test (GTT). **C)** Total cholesterol. **D)** Serum triglycerides. **E).** Alanine aminotransferase. **F)** Aspartate transaminase. Data presented as mean ± SEM. n=9-13 mice per group. ANCOVA was performed. HFD: high-fat diet; HFD+Ex: high-fat diet + exercise.

Supplementary Figure 7. Effects of exercise on PLIN5 protein levels. A) D) PLIN5 levels in the whole cell lysates in SHS. B) PLIN5 levels in whole cell liver lysates in NASH. C) PLIN5 levels in isolated LDs. D) PLIN5 levels in NASH mice under exercise. Data presented as mean $\pm$ SEM. n=4-5 per group. Student's t-test. Statistical significance $p < 0.05$; *among groups. CD: Control diet; Ex: exercise; HFD: High-fat diet; MCD: methionine-choline deficient diet.

Supplementary Figure 8. Exercise does not affect physical and biochemical parameters in the MCD-induced NASH model. A) Body weight. B) Food intake. C) Maximal speed. D) Serum insulin. E) ALT. F) AST. Data presented as mean $\pm$ SEM. n=8-4 by group. Student's t-test. MCD: methionine-choline deficient diet. MCD+Ex: methionine-choline deficient diet + exercise.

Supplementary Figure 9. PLIN5 overexpression has no role in the exercise-induced effects on SHS severity. A) Protocol design. B) PLIN5 protein levels in liver C) PLIN5 protein levels in heart, skeletal muscle, and brown adipose tissue (BAT). D) Transmission electron microscopy. E) LD-mitochondria interactions normalized to the total number of mitochondria. F) Body weight. G) Mean caloric intake. H) Liver weight. I) Maximal speed. J) Glucose tolerance test. K) Fasting glycemia. L) Insulin. M) Homeostatic Model Assessment of Insulin Resistance (HOMA-IR). N) Total cholesterol. O) HDL cholesterol. P) LDL cholesterol. Q) Triglycerides. R) Alanine aminotransferase. S) Aspartate transaminase. T) Liver H&E staining. Data presented as mean $\pm$ SEM. n=9-10 mice per group. Student's t-test. Statistical significance $p < 0.05$; *among groups.

Supplementary Figure 10. Aerobic exercise increases Mitofusin-2 in NAFLD. A) Mfn-2 levels in CM and PDM in the NASH model. B) Protocol design, Mfn-2 level in whole cell lysates, and representative micrograph of the liver. C) Lipid droplet size D) LD-mitochondria interaction normalized to the total number of mitochondria E) Circularity of PDM in WT and Mfn-2 L-KO models. Data presented as mean $\pm$ SEM. n=6-8 by group. Student's t-test or 2-way ANOVA, followed by Tukey's post-test were performed. Statistical significance $p < 0.05$; *among groups. MCD: methionine-choline deficient diet; MCD+Ex: methionine-choline deficient diet+exercise; CM: cytoplasmic mitochondria; PDM: peridroplet mitochondria. WT: Wild-type mice, Mfn-2 L-KO: liver-specific Mfn-2 knockout mice.

Supplementary Figure 11. Aerobic exercise increases phosphatidate levels. A) Binding of Mfn2 (1-613) expressed in *E. coli* to Membrane Lipid Strips. B) Phosphatidate in PDM. Data presented as mean SEM. n=5-8 per group. Student's t-test. Statistical significance $p < 0.05$; *among groups.

Supplementary Figure 12. Fatty acid composition in peridroplet mitochondria. Comparison of fatty acid composition (mean $\pm$ SEM) in the isolated mitochondria membrane from WT (A, C and E) and from Mfn2 L-KO (B, D, and F) mice. (A and B): Content of fatty acid families; (C and D): Content of individual saturated fatty acids (SFA) and (E and F): Content of individual monounsaturated fatty acids (MUFA). SFA: saturated fatty acid; MUFA: monounsaturated, fatty acids; PUFA: polyunsaturated fatty acids; n-6: omega-6; n-3: omega-3. Student's t-test or 2-way ANOVA were performed. Statistical significance $p < 0.05$, *among groups; $p < 0.001$, ***among groups; $p < 0.0001$, ****among groups.

Figure 1

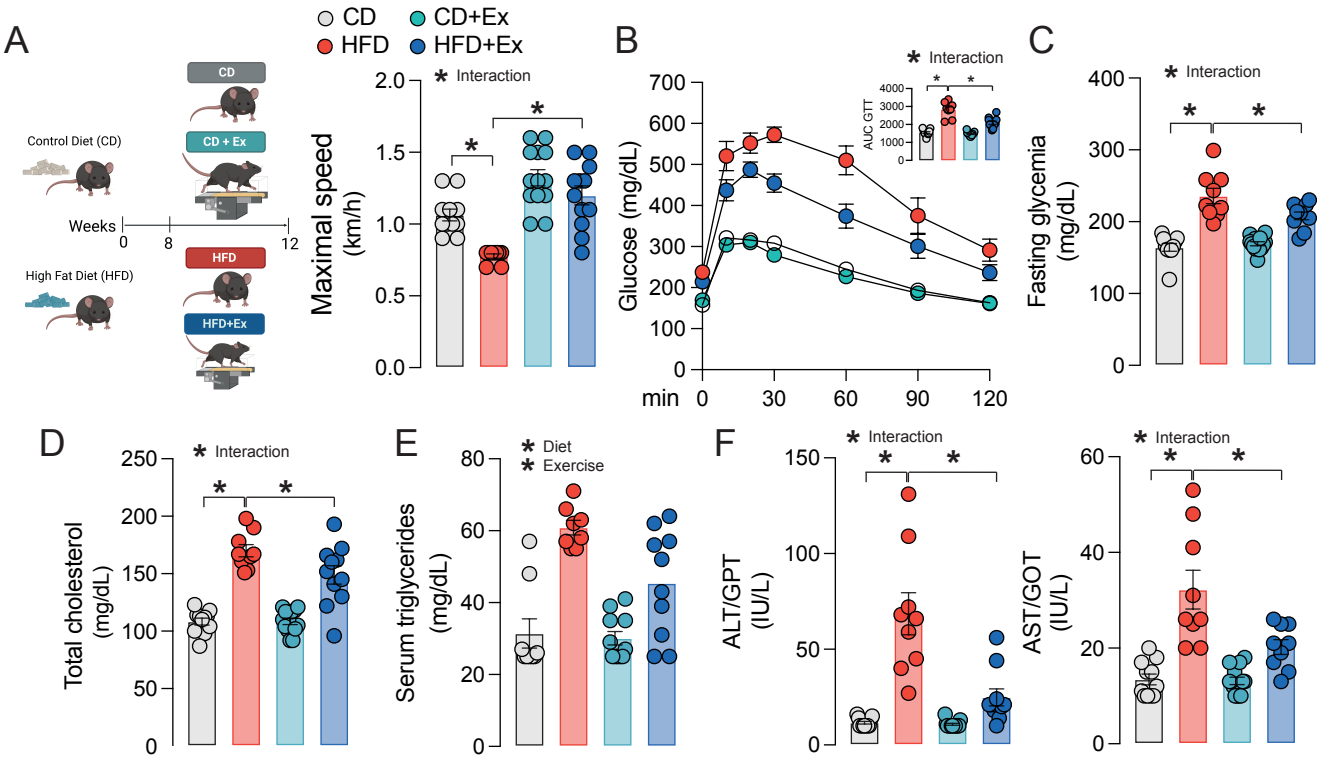


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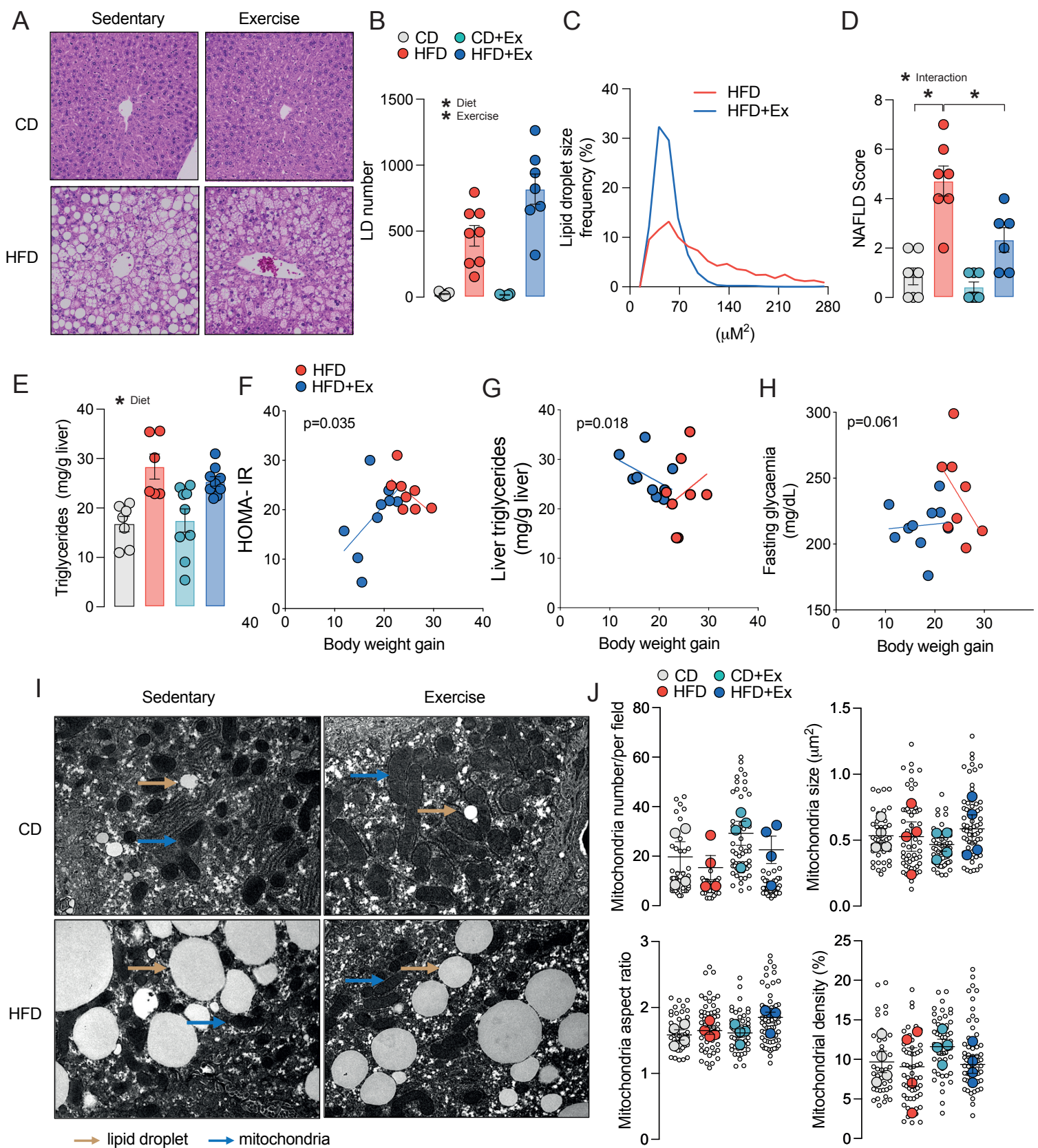


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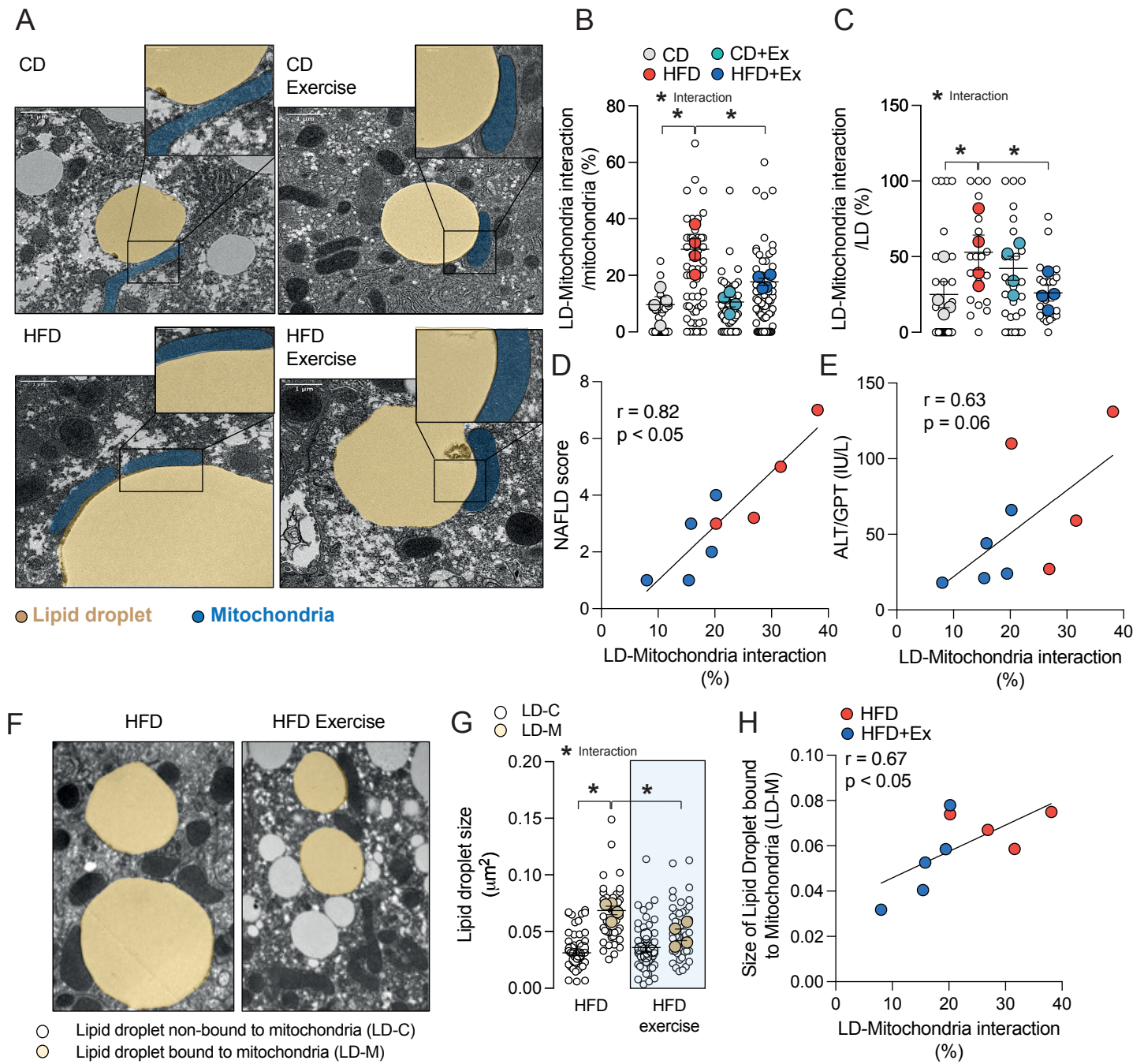


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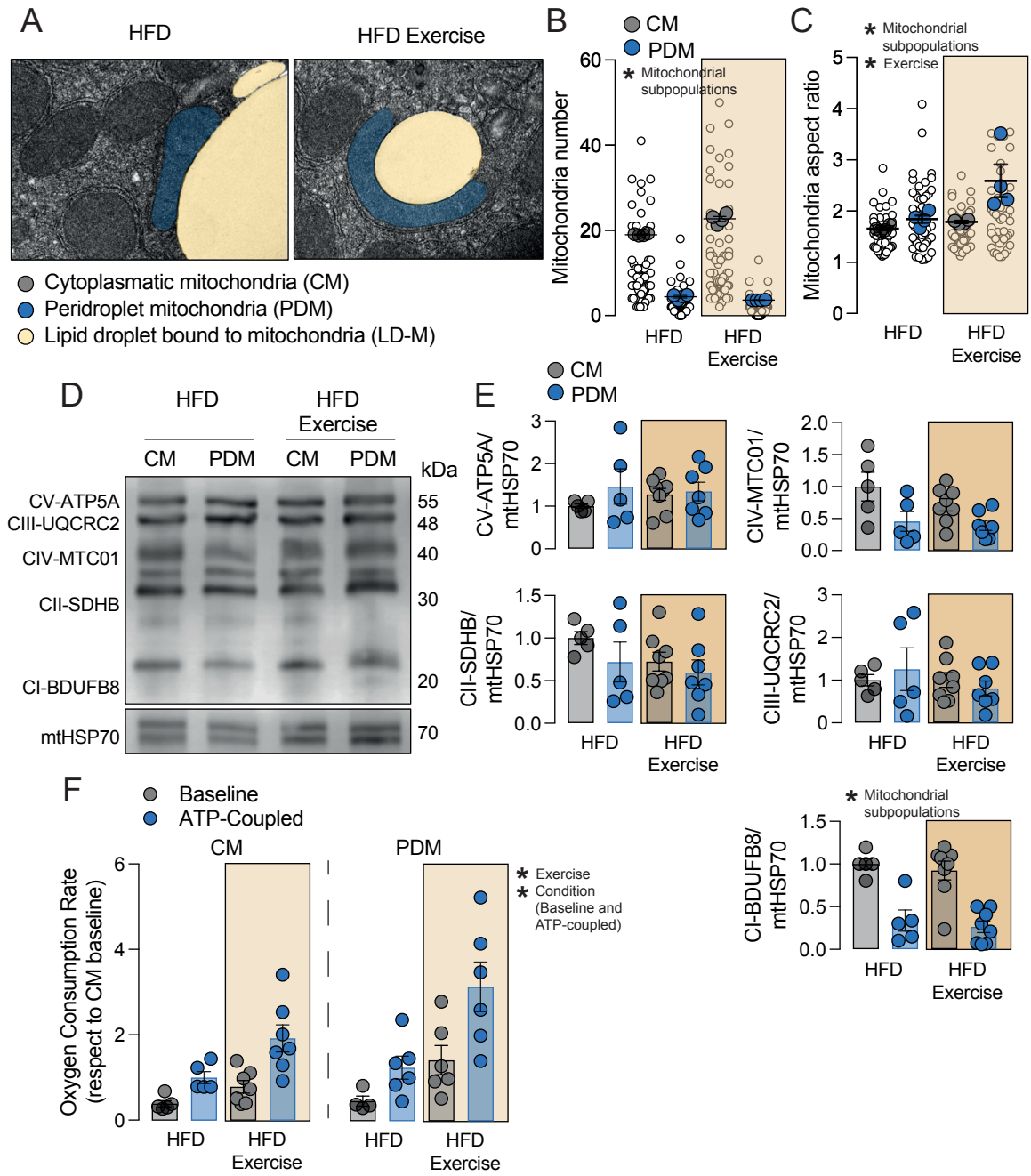


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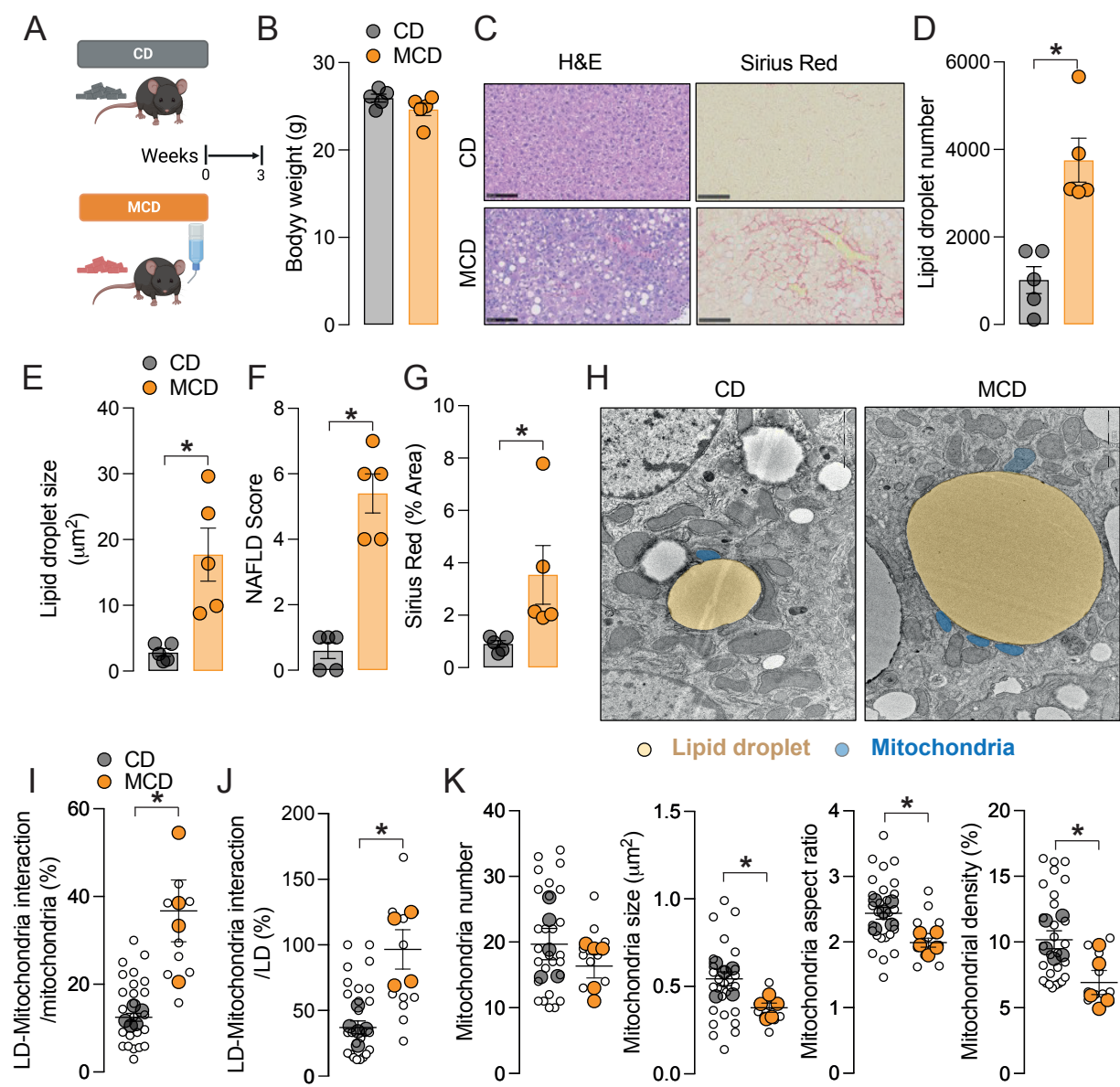


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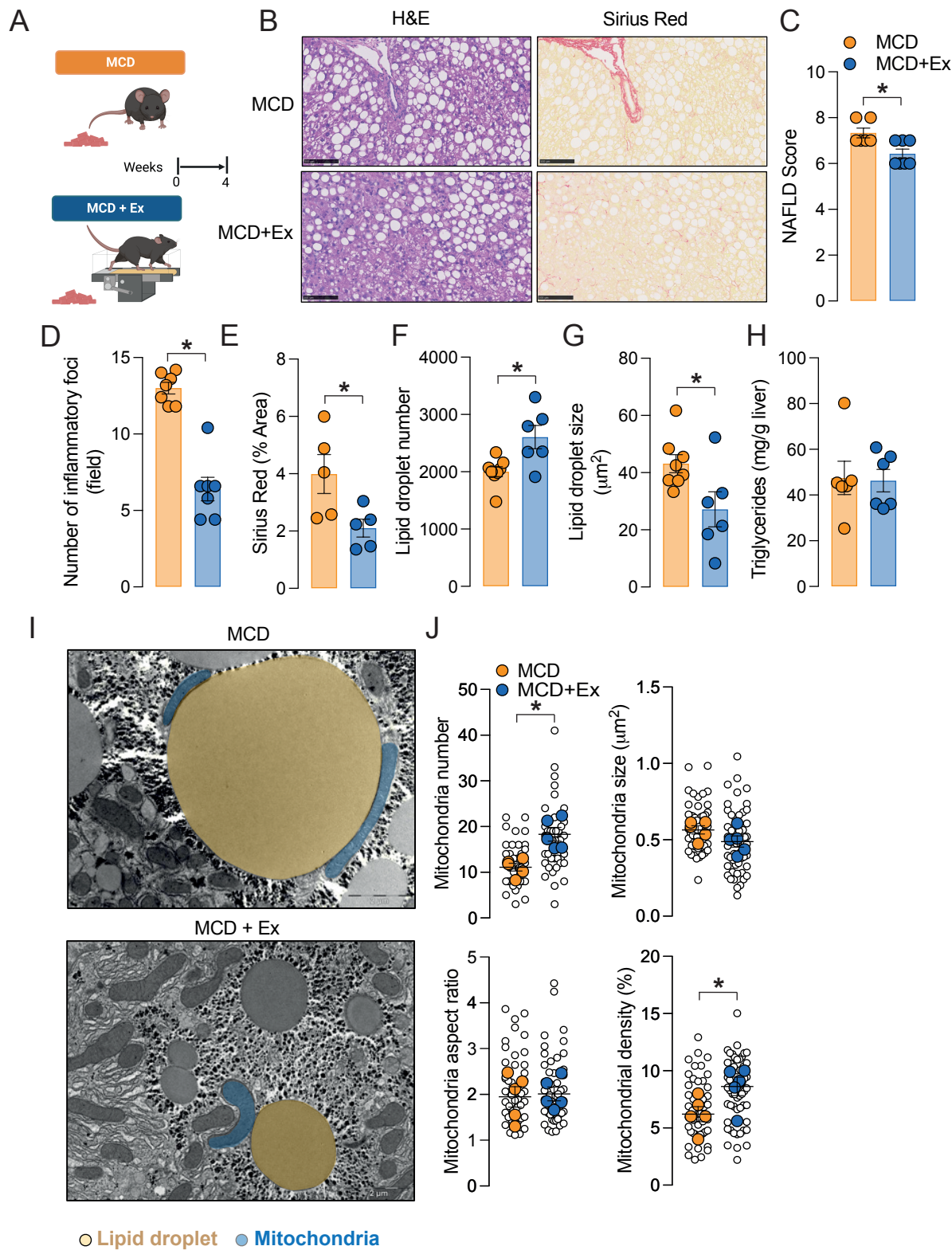


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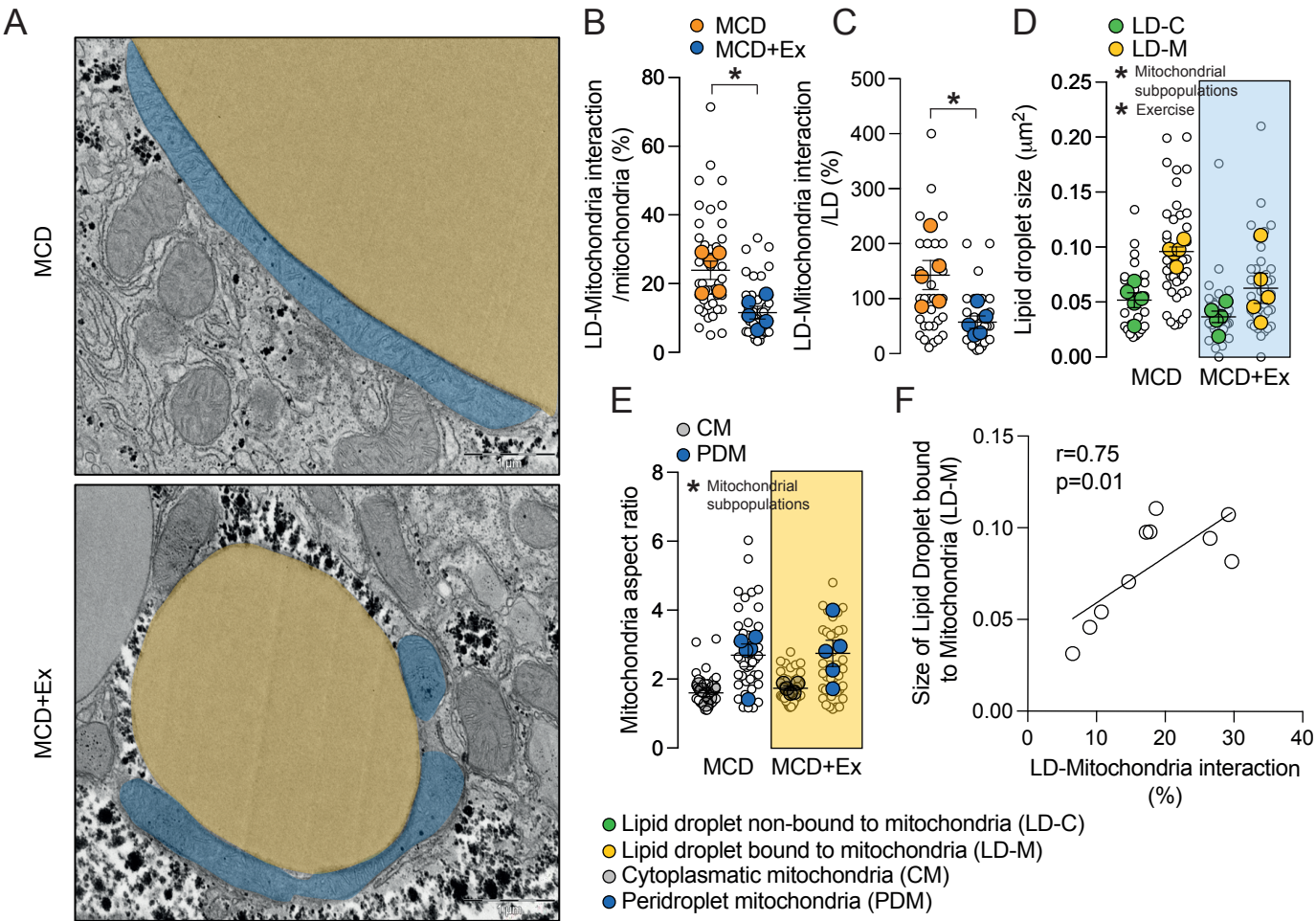
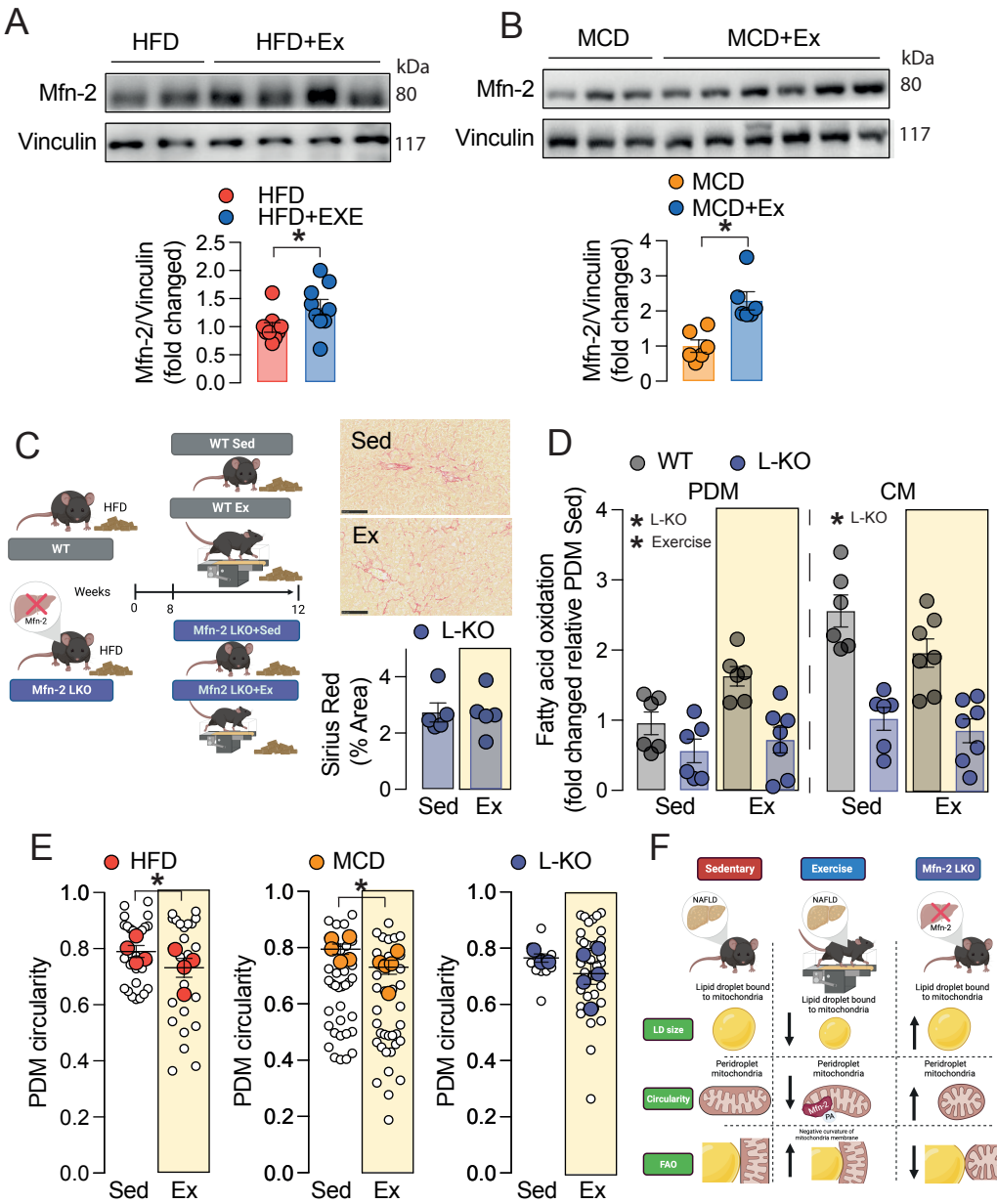


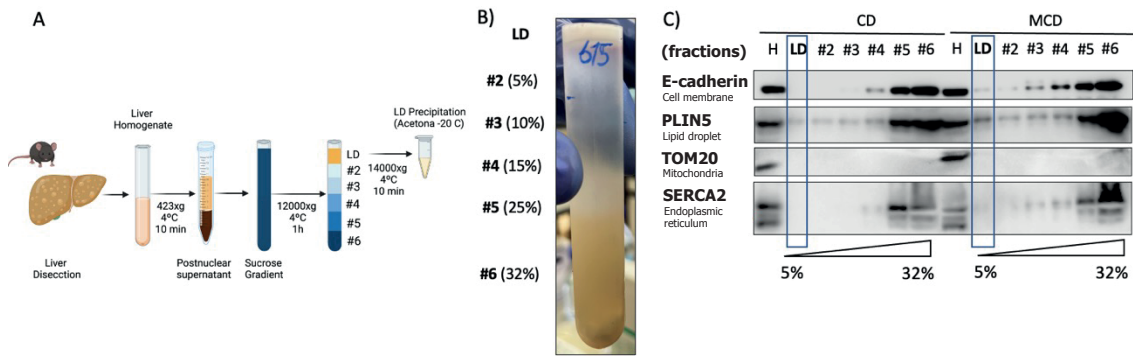
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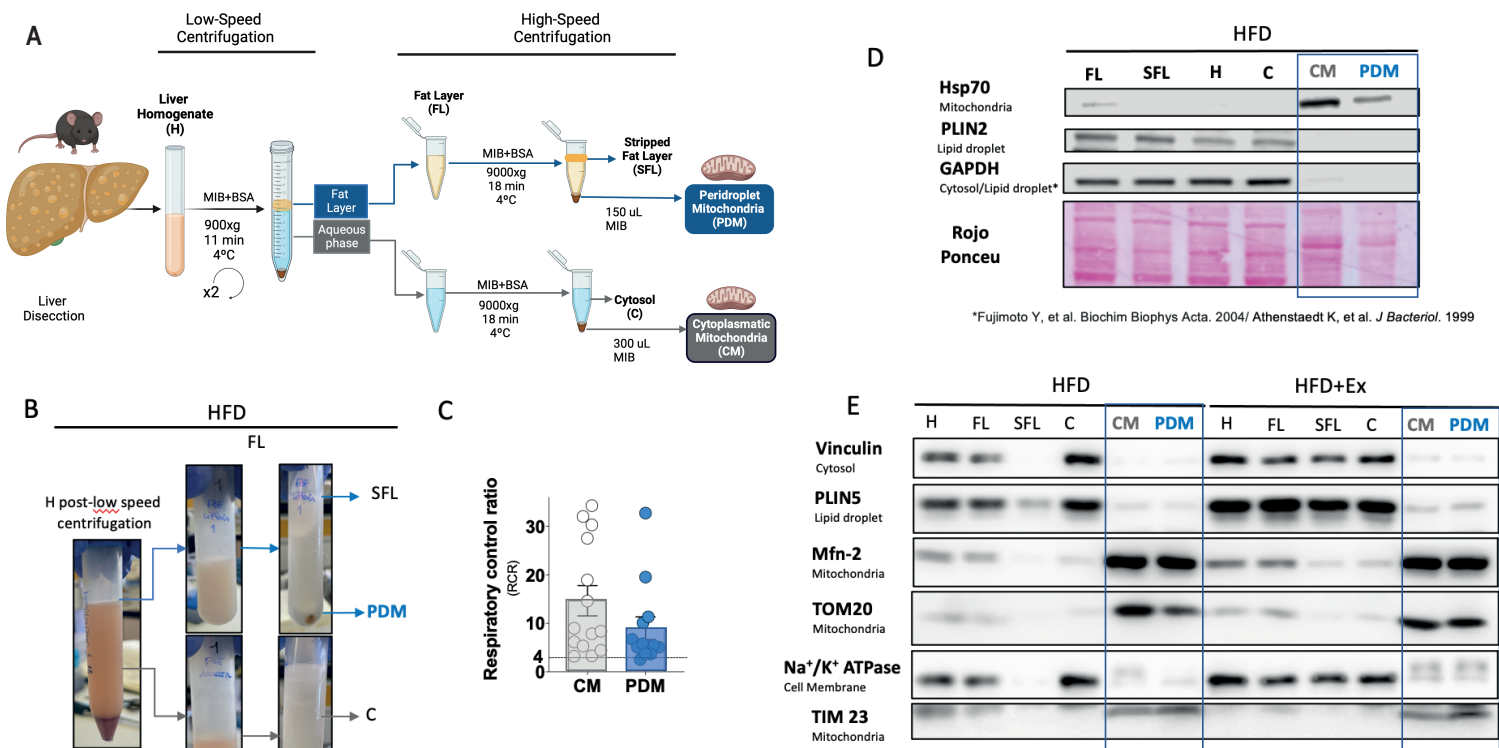
Credit authorship contribution statement

Juan Carlos Borquez conceived the study, performed experiments, analyzed the data and wrote the manuscript. **Francisco Díaz-Castro** performed experiments. **Francisco Pino-de la Fuente** performed experiments. **Karla Espinoza** performed experiments. **Ana María Figueroa** performed experiments. **Inma Martínez-Ruiz** performed experiments. **Vanessa Hernández** performed experiments. **Iliana López-Salgado** performed experiments. **Raúl Ventura** performed experiments. **Joan Carles Domingo** performed experiments and analyzed the data. **Marta Bosch** analyzed the data. **Alba Fajardo** performed experiments. **David Sebastian** performed experiments and wrote the manuscript. **Alejandra Espinoza** analyzed the data. **Albert Pol** analyzed the data. **Antonio Zorzano** analyzed the data and wrote the manuscript. **Víctor Cortés** conceived the study, analyzed the data, wrote the manuscript, and approved the final version of the manuscript for publication. **María Isabel Hernández-Alvarez** conceived the study, analyzed the data, wrote the manuscript, and approved the final version of the manuscript for publication. **Rodrigo Troncoso** conceived the study, analyzed the data, wrote the manuscript, and approved the final version of the manuscript for publication. All the authors contributed to editing.

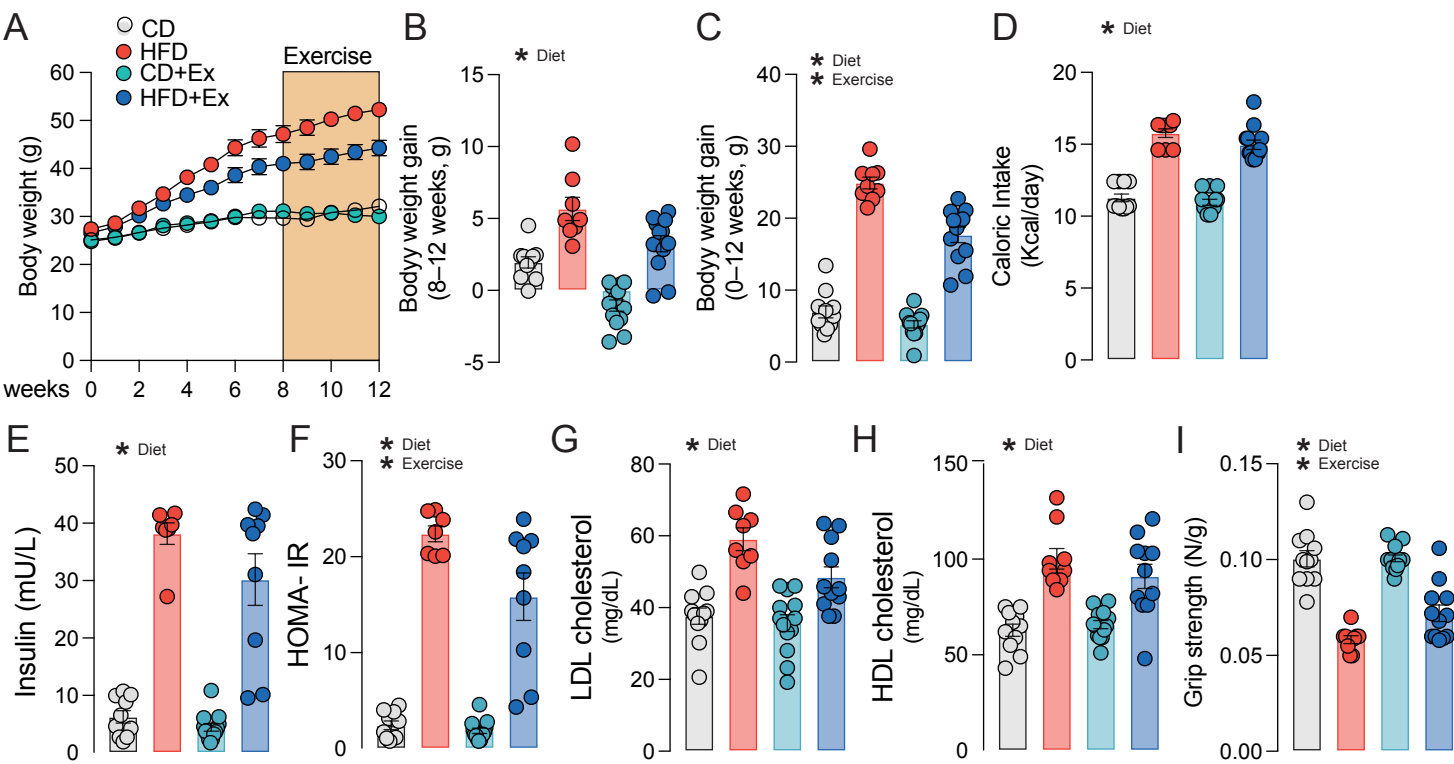
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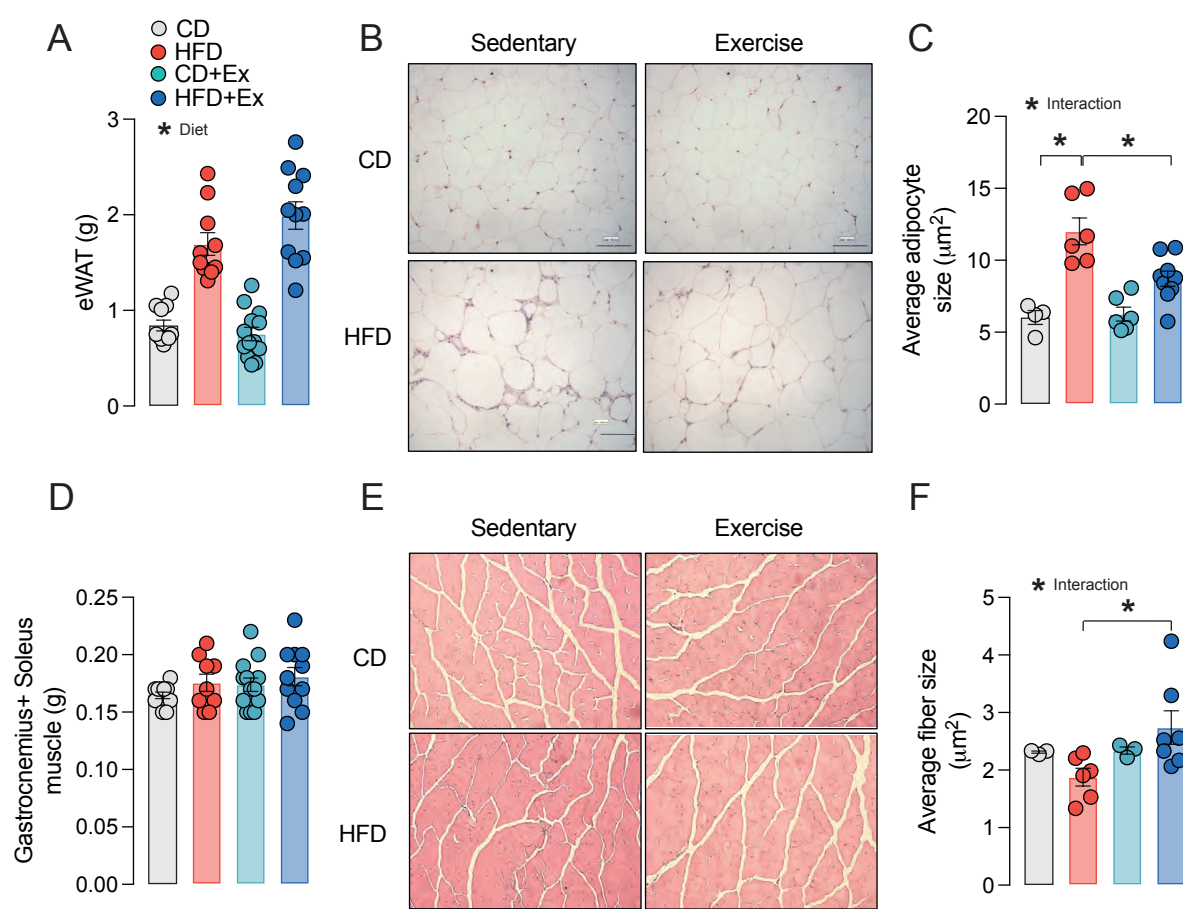
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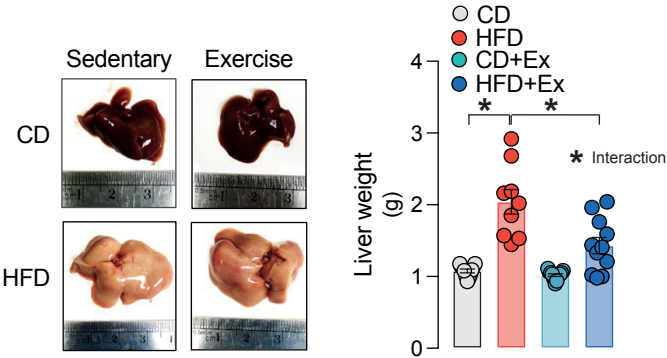
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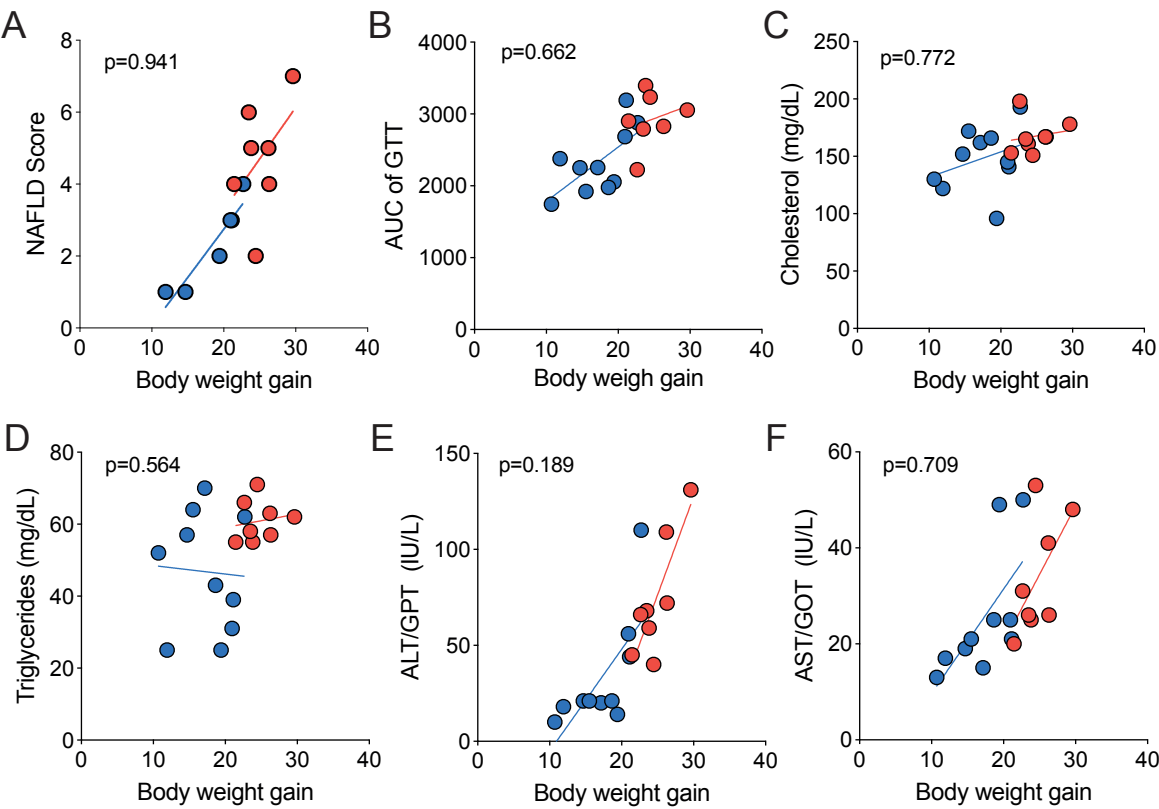
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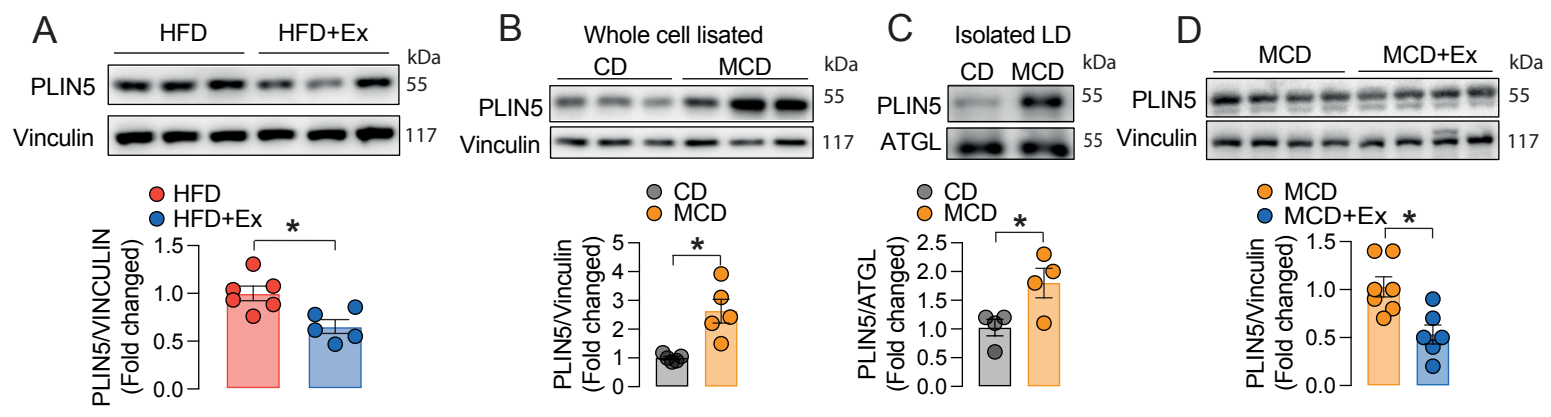
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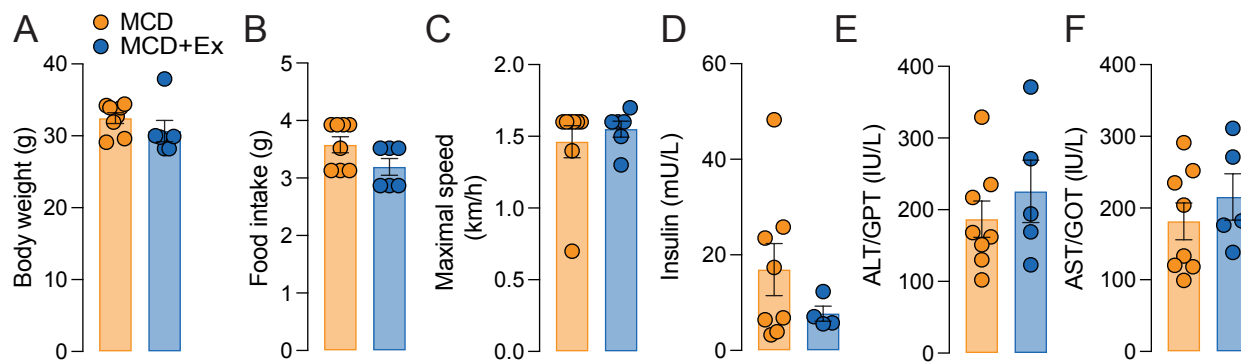
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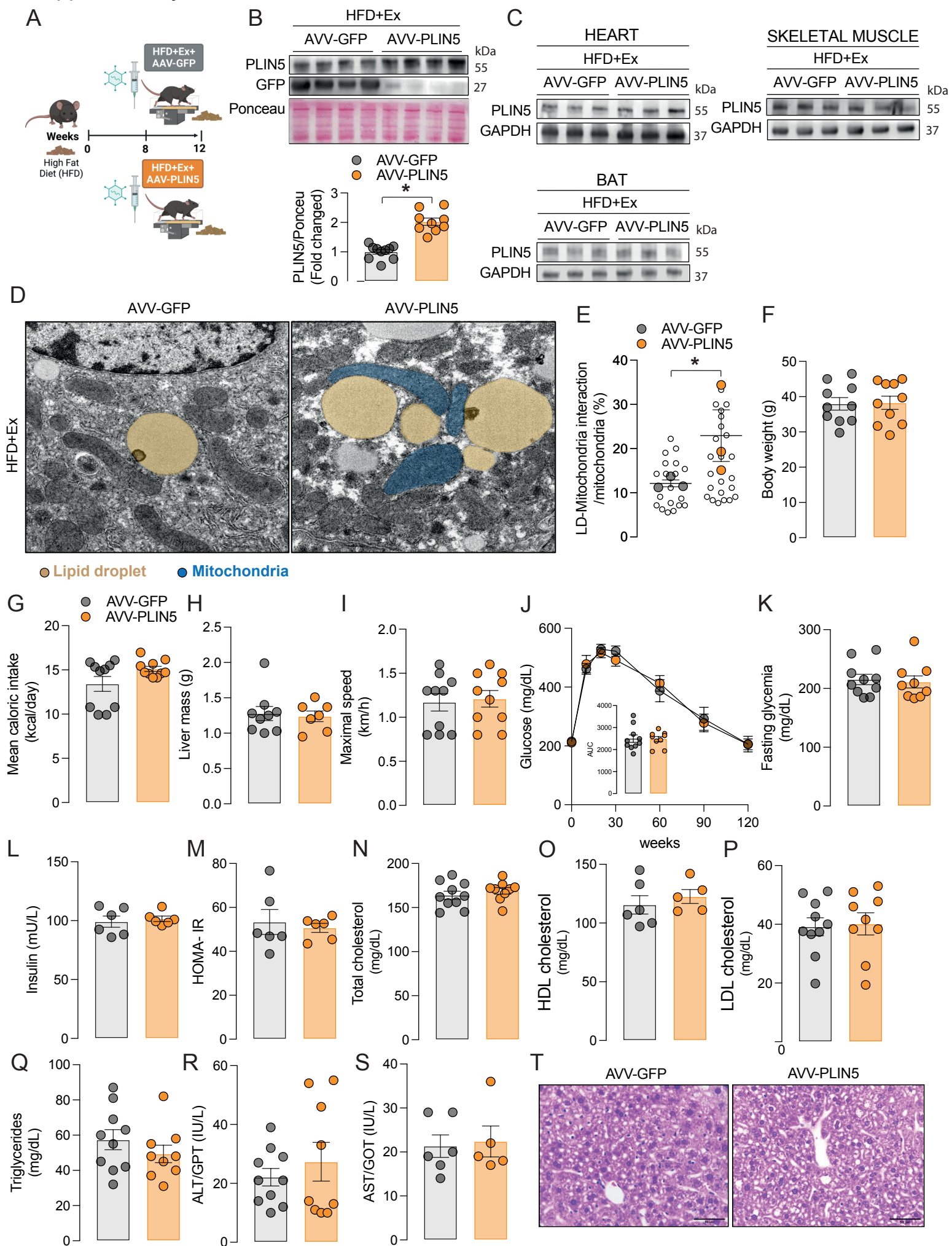
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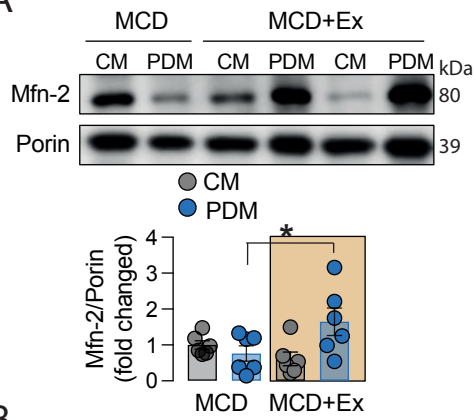


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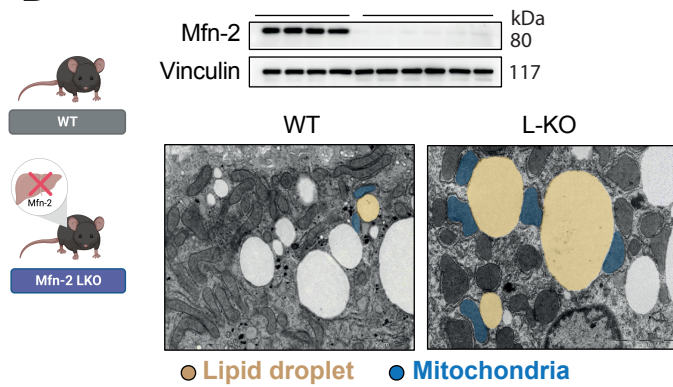


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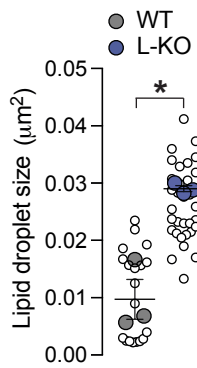
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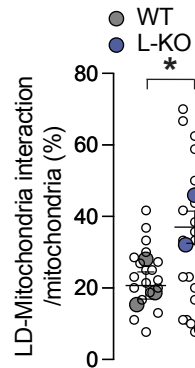
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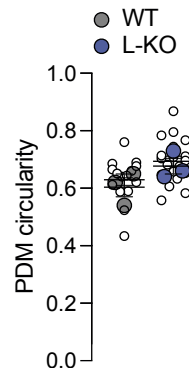
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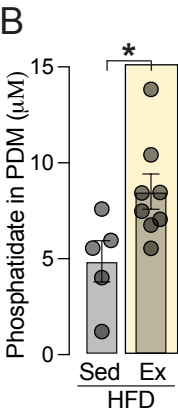
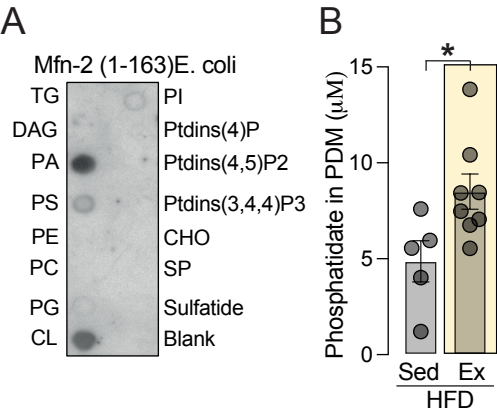
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E



Supplementary 11



Supplementary 12

