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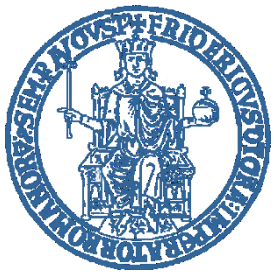

DOTTORATO DI RICERCA

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TERAPIE AVANZATE BIOMEDICHE E CHIRURGICHE

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Regular exercise prevents the transition from fatty liver to steatohepatitis

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Regular exercise prevents the transition from fatty liver to steatohepatitis

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) affects about 25% of the world population [1] and its burden on society is projected to increase in the next decades [2]. Clinically, NAFLD covers a spectrum of conditions ranging from non-alcoholic fatty liver (NAFL) to decompensated liver cirrhosis, which can progress to hepatocellular carcinoma (HCC). NAFL is defined by steatosis with or without inflammation but without ballooning injury. In contrast, non-alcoholic steatohepatitis (NASH) is defined by the presence of ballooning injury with inflammation in addition to steatosis [3]. The transition from fatty liver to NASH is an important milestone in the evolution of the disease. In a prospective study from the NASH clinical research network, changes in the NASH activity score, which includes ballooning injury as a marker, was associated with concordant changes in fibrosis [4]. In turn, the degree of hepatic fibrosis is related to both liver-related mortality of NAFLD and to the overall mortality [5]. Currently there is no pharmacological treatments that can arrest the progression of NAFL to NASH or reverse NASH once it is established, although several experimental drugs are being tested [6].

NAFLD, in general, and NASH, in particular, are associated with high caloric, high fat diet and sedentariness. Hence, NAFLD is linked to a

metabolic overload of the liver. Consequently, clinical trials proposing weight loss and other lifestyle interventions as therapeutic measures have enrolled patients with NASH and fibrosis and sought to demonstrate the resolution of NASH and the reversal of fibrosis. In general, weight loss is positively correlated with an improvement in histologic features of NASH, but for a resolution of NASH and regression of fibrosis, weight loss must be superior to 10% [7-8]. One specific lifestyle intervention documented to improve the hepatic metabolic situation is regular physical activity [9]. In fact, physical activity reduces steatosis in patients with NAFLD even when it is not associated with weight loss [10-11]. Moreover, resistance exercise in sedentary adults improves steatosis without impact on body weight [12]. Nonetheless, the interpretation of these clinical studies is confounded when dietary and exercise interventions cannot be dissociated. Whether selective intervention of an exercise regime in patients who maintain their high fat diet is beneficial and whether exercise alone can impede the transition from NAFL to NASH and thus prevent the evolution to fibrosis are uncertain. Also unclear is which signaling pathways are operative in transducing the beneficial effects of exercise when the NASH-inducing diet remains in place.

We previously reported that in a PTEN knockout, genetic mouse model of NASH, regular physical activity decreases the incidence HCC but without a change in the NAFLD activity score (NAS) score [13]. We also reported that in a rat model of orthotopic syngeneic tumour implantation, regular physical activity downregulated the expression of hepatic genes associated with the development of HCC [14]. However, it remains unknown whether an exercise regimen can intervene at all points, or only at specific points, along the continuum from NAFL to NASH to fibrosis and HCC and change the course of the disease, especially within the context of continued nutritional overload. To explore the selective benefits of physical activity in NAFLD triggered by a high-fat diet and to elucidate its mechanism, we queried whether daily exercise alone halts the transition from NAFL to NASH and impairs the progression of fibrosis. We chose a choline-deficient, high-fat diet mouse model that displays a progressive phenotype of NAFL to NASH to hepatic fibrosis and eventually HCC and compared the disease outcome in sedentary and exercised mice.

METHODS

Study design and animals

Male C57Bl/6N mice (Charles River, Frieburg, Germany) were chosen to avoid the Nnt (nucleotide nicotinamide transhydrogenase) mutation carried by C57BL/6J mice. Loss of NNT enzymatic activity has been linked to reduced mitochondrial NADPH/NADP+ ratio, mitochondrial redox abnormalities [15], as well as impaired mitochondrial peroxide metabolism [15] and glucose homeostasis [16]. Mice aged 8 weeks were housed under controlled temperature (22 ± 2°C) and lighting (12h light-dark cycles), acclimatized to the facility for one week, then randomly assigned to one of the following four groups and subjected to a diet and activity protocol (Fig. 1): 1) mice (n=11) fed a standard diet for 12 weeks (control group); 2) mice (n=11) fed a choline deficient - high fat diet (CD-HFD) for 12 weeks (baseline NAFL group); 3) mice (n=11) fed CD-HFD for 20 weeks but without exercise (CD-HFD sedentary group); mice (n=11) fed CD-HFD for 20 weeks but with treadmill exercise from weeks 13 to 20 (CD-HFD exercise group) (Fig. 1). Food intake and body weight were monitored weekly. Mice received humane care and experiments were approved by the Committee for Animal Use, Canton of Bern, Switzerland.

Dietary intervention

All mice were fed ad libitum. The CD-HFD contained 9% protein, 60% fat including 2% cholesterol and 31% carbohydrate (HF-CDAA diet, E15673-94, Ssniff Spezialdiäten GmbH, Germany). The standard chow diet contained 12% protein, 16% fat and 72% carbohydrate (Control diet, E15668-04, Ssniff Spezialdiäten GmbH, Germany).

Exercise protocol

After 12 weeks, the CD-HFD exercise mice were placed on a treadmill (running speed of 12.5 m/min) (Förderband GFB, Elmotec, Kleindöttingen, Switzerland) for 60 minutes from 08.00 h to 09.00 h, corresponding to their waking time. Exercise was imposed 5 days/week for 8 weeks. Sedentary mice remained in their cages.

Animal euthanasia

The control and baseline NAFL groups were killed after 12 weeks. The CD-HFD sedentary and exercise groups were killed after 20 weeks, 2 days after the last exercise session. Mice were anesthetized with pentobarbital (100 mg/kg i.p.) and blood was collected from the inferior vena cava into heparinized tubes then centrifuged (3000 × g, 15 min, 4 °C). Plasma was stored at -80 °C for less than 1 month. Before

anesthesia, tail blood lactate and glucose levels were measured with a Lactate Scout Analyzer (Senslab, Leipzig, Germany) and an automated glycemia reader (Ascensia Contour, Bayer Health Care, Zürich, Switzerland). After euthanasia, liver tissue was divided and either immediately weighed and snap-frozen in liquid nitrogen or placed in RNAlater (Sigma-Aldrich R0901) and stored at -80°C or fixed in 4% phosphate buffered formaldehyde. Hepatic tumors were counted, sized and fixed.

Plasma analyses

Activity of alanine transaminase (ALT) and aspartate transaminase (AST), and concentrations of triglycerides, total cholesterol, total bilirubin and bile acids were measured (Cobas analyzer 8000, Roche Diagnostics GmbH, Mannheim, Germany). PRO-C3, the N-protease mediated cleavage of the N-terminal propeptide of type III collagen, PRO-C4, an internal epitope in the 7S domain of type IV collagen, and C6M, a neo-epitope of the proteolytic degradation of type VI collagen, were measured by means of a competitive enzyme-linked immunosorbent assay (Nordic Bioscience, Herlev, Denmark), as described [17].

Histology

Formaldehyde-fixed, paraffin-embedded liver tissue were stained with hematoxylin and eosin (H&E) and examined for steatosis, NASH lesions and tumors, by a pathologist blinded to treatment conditions (LMT). The NAS score was determined as previously defined by Kleiner et al [18]. The degree of fibrosis was assessed on sections stained with Sirius Red and visualized with a using panorama scanner and case viewer (3D Histech) and 10x objective. Eight digital images were collected from different areas of the left, median and right lobes and the signals were quantified with the MetaMorph® image analysis software (Molecular Devices, Sunnyvale, CA). Tumor types were assessed as previously described [19].

Hepatic triglycerides and free fatty acids

Total triglyceride content was measured with the PicoProbeTM triglyceride fluorometric assay (BioVision, Milpitas, CA). Total free fatty acid content was quantified by means of the fluorometric FFA kit (BioVision).

Tissue lysis and immunoblot analysis

Livers were homogenized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS and 50 mM Tris-HCl pH 7.4) containing protease and phosphatase inhibitors (Roche, Rotkreuz, Switzerland). Protein concentration was measured with the PierceTM BCA assay (Thermo Fisher Scientific, Rockford, IL). Equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes, blocked for 1 hour with 5% nonfat milk or BSA, then incubated overnight at 4°C with primary antibodies purchased from Cell Signaling Technology (Leiden, The Netherlands), Proteintech Group, (Manchester, UK), Abcam (Cambridge, UK), or Thermofisher (Waltham, MA, USA). Detection of vinculin (Merck KGaA, Darmstadt, Germany) served as the loading control. After incubation with peroxidase-conjugated secondary antibody (Thermo Fisher Scientific, Rockford, IL), signals were revealed with enhanced chemiluminescence (Amersham ECL Prime, GE Healthcare, Glattburg, Switzerland) and a Fusion CCD camera coupled to a computer equipped with Fusion Capt Fx Software (Vilber-Lourmat, Marne-la-Vallée, France). Signals were quantified with the Bio-1D Advanced software (Vilber-Lourmat).

Isolation of total RNA and quantitative PCR

Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland) and stored at -80°C. RNA was reverse-transcribed (SuperScript III Reverse Transcriptase, Invitrogen, Basel, Switzerland). The gene primer, FAM-labelled probe and the TaqMan Universal PCR Master Mix were obtained from Applied Biosystems. Amplification was performed with an ABI PRISM 7500 Sequence Detection System. The ΔCt values in triplicate were calculated relative to β2-microglobulin as the housekeeping gene. Values are reported as fold increase or decrease relative to the controls and calculated as $2^{-\Delta\Delta Ct}$.

Scanning Electron Microscopy

Livers were perfused through the portal vein with fixation solution (2.5% glutaraldehyde, 2% formaldehyde, 2 mM CaCl2, 2% sucrose and 0.1 M sodium cacodylate (pH 7.4)) for 5 min. Fixed tissue was cut in blocks (1mm*1mm*5mm) and stored in 2% formaldehyde at 4ºC until processed.

For Scanning Electron Microscopy (SEM), fixed livers were treated with 1% osmium tetroxide, dehydrated in a graded series of ethanol, and dried. The sections were coated with platinum/palladium and visualized

under an S-4700 electron microscope (Hitachi). For evaluation of capillarization, the percent of open space area in the liver sinusoidal endothelial cells (LSECs) (porosity) was measured in 15 randomly selected fields at ×10000 magnification on at least three animals per group, using ImageJ software.

Respiration in isolated liver mitochondria

Oxygen flux was measured in freshly isolated mitochondria by respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria). Mitochondria (200 μg) were added to 2 ml of respiration buffer [110mM sucrose, 60mM K+-lactobionate, 0.5mM EGTA, 3 mM MgCl2, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES (pH 7.1), at 37°C] [20]. Oxidative phosphorylation was estimated with complex I (pyruvate 5mM, malate 2mM, glutamate 5mM) and complex II (succinate 10mM) substrates in the presence of ADP (2.5 mM). Leak respiration was recorded after addition of oligomycin (2.5 μM). For maximum uncoupled respiration, the protonophore carbonyl cyanide m-chlorophenyl hydrazine (CCCP) was titrated in 0.5 μM increments until maximal stimulation of respiration. The protocol was terminated by assessing non-mitochondrial respiration with the complex I and III inhibitor, rotenone (0.5 mM) and antimycin A (2.5 mM), respectively.

Finally, the activity at complex IV was recorded with the artificial substrate N,N,N9,N9-tetramethyl-pphenylenediamine dihydrochloride (TMPD) (0.5 mM) and ascorbic acid (2 mM), and inhibited with azide (100mM). The cytochrome c control factor was measured after simulation respiration with exogenous cytochrome c (10 μM). Respiration states were corrected for non-mitochondrial respiration and complex IV activity was corrected for azide inhibition. Values were normalized for protein as described previously [20].

Statistical analysis

Data are presented as the mean values ± standard deviations (SD). The normality of data was assessed by the Kolmogorov-Smirnov test. The nonparametric Mann-Whitney U test was applied in the case of non-normal distributions. The Fischer's exact test was applied for frequency tables. A p value $\leqslant 0.05$ was considered statistically significant.

RESULTS

Exercise attenuates the transition from NAFL to NASH

We initially assessed the effect of CD-HFD and exercise on liver lesions by grading the degree of steatosis. Compared to control mice, all CD-HFD-fed mice developed extensive steatosis (>90% steatosis, grade 3) (Fig. 2a). This finding was evident in both the early 12 weeks CD-HFD group and in both the sedentary and exercise 20 weeks CD-HFD groups, which showed extensive macrovescicular and small droplet steatosis. Hepatocellular ballooning, a requisite feature for diagnosing NASH, was absent in 12 weeks CD-HFD mice. However, ballooning was present after 20 weeks of CD-HFD but was significantly attenuated by exercise. Ballooning was observed in all 8 mice of the sedentary group (grade 2 in 90% of mice) predominantly in zone 3, whereas it was recorded in only 28% of the exercise group (Fig. 2b). Neither Mallory-Denk bodies nor apoptotic bodies were detected in either groups. Consistent with the development of NAFL and NASH, all CD-HFD fed mice showed hepatic inflammation (grade 3). The NAS score increased significantly in the sedentary mice when compared to the NASH baseline and to the exercised mice (Fig. 2c).

Exercise halts the progression of fibrosis

To investigate the influence of exercise on liver fibrogenesis, we assessed total collagen in the liver and quantified circulating levels of three biomarkers, Pro-C3, Pro-C4 and C6M in the plasma. After 12 weeks, NAFL mice displayed diffuse pericellular fibrosis but no portal fibrosis (Fig. 3a). After 20 weeks, sedentary mice displayed even more fibrosis, but this change was halted by exercise (Fig. 3b). In fact, the extent of fibrosis in the exercised mice was comparable to that observed in 12 weeks NAFL livers. Pro-C3, the proteolytic propeptide released during formation of type III collagen, was significantly elevated after 12 weeks of CD-HFD, increased further after 20 weeks of CD-HFD in sedentary mice but this rise was halted in exercised mice (Fig. 3c). Another biomarker, Pro-C4, which reflects synthesis of the basement membrane collagen, type IV, was less affected by early CD-HFD. However, it increased after 20 weeks of CD-HFD in sedentary mice, a rise that was prevented by exercise (Fig. 3c). C6M, a degradation marker for type VI collagen, was slightly increased after 12 weeks CD-HFD, was significantly increased 2.5-fold after 20 weeks CD-HFD in sedentary mice, but this rise was prevented by exercise (Fig. 3c). Thus,

the changes in biomarkers were congruent with the changes observed in histology.

Exercise improves biochemical markers of NAFLD in plasma

Biochemical markers, indicative of liver disease, were measured in the plasma. Concentrations of both transaminases, ALT and AST, were increased at 12 weeks and further increased after 20 weeks of CD-HFD, but only in sedentary mice (Fig. 4). The increase in ALT and AST concentrations from 12 to 20 weeks was halted in exercised mice. Total bile acids increased after 12 weeks of CD-HFD but the differences between sedentary and exercised mice at 20 weeks were not statistically significant. Exercise tended to lower plasma triglycerides. Cholesterol increased modestly after 12 weeks of CD-HFD and tended to be lower at 20 weeks in the exercised group.

Exercise decreases hepatic triglyceride content

The histological changes elicited by exercise showing reduced steatosis were confirmed with biochemical measurements (Fig. 5a). After 12 weeks on CD-HFD, hepatic levels of triglycerides rose 1000-

fold. A further increase at 20 weeks was evident only in sedentary mice, whereas the levels remained constant in exercised mice. The hepatic levels of free fatty acids (FFAs) showed a different pattern. After 12 weeks, levels increased but thereafter remained stable and did not change with exercise (Fig. 5a). In all mice fed the CD-HFD, the mRNA level of the scavenger receptor and lipid transport facilitator, CD-36, was elevated at least six-fold and was unaffected by exercise (Fig. 5b). However, the expression of the long chain fatty acid carriers, fatty acid transport protein (FATP) FATP2 and FATP5 showed the reverse trend (Fig. 5c). The mRNA of FATP2 and FATP5 were both decreased by the CD-HFD. Exercise only partially reversed this trend for FATP2.

The hepatic expression of lipogenic enzymes was decreased by the CD-HFD (Fig. 5d). After 12 weeks, both fatty acid synthase (FAS) and ATP citrate lyase (ATPCL) decreased significantly. Moreover, the phosphorylation of ATPCL was decreased and exercise also decreased the ratio of P-ATPCL/ATPCL relative to sedentary livers. The hepatic lipid catabolic enzymes were also evaluated. CD-HFD decreased the levels of hormone-sensitive lipase (HSL) but the balance of phosphorylation at sites 563 relative to 565 was changed by exercise. The expression of perilipin 2 was decreased and remained depressed by

the CD-HFD. In addition, the expression of adipose triglyceride lipase (ATGL), which in hepatocytes augments fatty acid oxidation was lowered in all CD-HFD fed livers (Fig. 5d).

Exercise modulates TNFα

The mRNA expression of several markers of NAFLD was measured in liver. The expression of the pro-inflammatory cytokine tumor necrosis factor (TNF)α increased after 12 and 20 weeks of CD-HFD in comparison to controls, but exercise tended to decrease the levels (Fig. 6a). The expression of transforming growth factor (TGF)β1 was similarly elevated in all CD-HFD treated livers (Fig. 6b). However, exercise did not influence its expression. Insulin-like growth factor 2 (IGF-2) was significantly elevated in the sedentary NASH group relative to all other groups (Fig. 6c).

CD-HFD induces ER stress

Selected markers for endoplasmic reticulum (ER) stress were examined by immunoblotting in homogenates of liver. Binding immunoglobulin protein (BiP) tended to decrease in the exercised group (Fig. 7). After 12 weeks of CD-HFD, the expression of X-box binding

protein 1 (XBP-1s), which results from inositol-requiring enzyme (Ire)1 mediated splicing of XBP-1 mRNA, was severely downregulated. Levels of XBP-1s remained depressed at 20 weeks in both exercised ad sedentary groups. Conversely, after 12 weeks of CD-HFD, the expression of CCAAT-enhancer-binding protein homologous protein (CHOP) was induced. This upregulation was even more pronounced after 20 weeks of CD-HFD and was not affected by exercise (Fig. 7). Since CHOP can trigger the intrinsic apoptotic pathway, we quantified the expression of the pro-apoptotic protein, BAX, and the anti-apoptotic protein, BCL2. The CD-HFD was associated with an upregulation of BAX such that the ratio of BAX to BCL2 increased significantly (Fig. 7). Exercise tended to decrease this ratio.

Exercise increases autophagy

To evaluate whether the autophagy activation in the liver contributes to the improvement in hepatic steatosis after exercise, we evaluated the level of autophagy-specific microtubule-associated protein light chain 3 (LC3) by immunoblotting of liver homogenate in the exercised and sedentary CD-HFD mice. The analysis of LC3BII and LC3BI demonstrated a significantly higher ratio of LC3II / LC3I in the exercised group, which is indicative of an accumulation of

autophagosomes ($p \leq 0.005$, Fig. 8). Since mTOR acts upstream to inhibit autophagy, we evaluated the expression of mTOR and its phosphorylation at S2448, which correlates with the activity of mTORC1. Both the expression of mTOR and P-S2448-mTOR were lower in livers from exercised mice than in those of sedentary mice (Fig. 8). To evaluate selective autophagy in the mitochondria, we tested for the recruitment of active, phosphorylated PTEN-induced kinase (PINK) to the mitochondrial compartment. P-Pink was higher in the exercise group (Fig. 8).

Exercise decreases hepatic nodules formation

Extended exposure to a CD-HFD can lead to hepatic tumors. After 20 weeks, all mice in the sedentary CD-HFD group indeed displayed liver nodules, histologically compatible with hepatocellular adenoma (Fig. 9a). The incidence of tumor nodules was significantly reduced in the exercised group, wherein 70% of mice developed liver nodules ($p < 0.01$). Furthermore, exercise negatively affected tumor burden; the mean number of nodules per liver was reduced by 2.9 ± 2.5 *vs* 7.7 ± 4.4 for sedentary and exercise, respectively ($p < 0.01$) (Fig. 9b). To elucidate the mechanism mediating these tumor suppressive effects of exercise, we

examined the AMP-activated protein kinase (AMPK) – mTOR signaling pathway in liver homogenate. First, we quantified the phosphorylation of AMPKα on T172, as a measure of AMPK activation, then the activating phosphorylation of S6, responsible for ribosomal biogenesis and translation (Fig. 9c). After 20 weeks of CD-HFD, phosphorylation of AMPK (T172) increased whereas phosphorylation of S6 decreased in exercised mice (Fig. 9c).

Exercise does not change mitochondrial respiration

Since dysfunctional mitochondria contribute to the progression of NAFLD, we queried whether exercise halted the progression of NAFL to NASH by improving mitochondrial respiration. In respirometry studies, mitochondria from NAFL livers showed no difference in combined complex I and II driven respiration but displayed a higher leak respiration, an indicator of dissipated membrane potential, and a significantly lower maximal respiration and complex IV respiration than did control mitochondria (Fig. 10a). These differences were not detected when NASH sedentary and NASH exercise groups were compared. However, the cytochrome *c* control factor was significantly increased relative to control in all NAFL and NASH groups (Fig. 10b). In addition,

citrate synthase (CS) enzyme activity and expression were monitored as an indicator of the tricarboxylic acid cycle, TCA (Fig. 10 c, d). Whereas the protein expression of CS remained constant in all groups, the activity increased in NAFL relative to control (Fig. 10c) but not in exercise relative to sedentary. To determine whether a decrease in cytochrome *c* content could account for the increase in cytochrome *c* control factor, we quantified the protein expression in mitochondria. Expression of cytochrome *c* decreased in NAFL relative to controls but remained constant in NASH sedentary relative to exercise (Fig. 10d). To test whether the decrease in maximal respiration and complex IV respiration could be attributed to a downregulation of respiratory proteins, we quantified the expression of COX IV. COX IV was significantly lower in NAFL than in controls. The expression was similar in NASH sedentary and exercise groups (Fig. 10d).

The CD-HFD promotes sinusoidal endothelial cell defenestration

After 12 weeks of CD-HFD, the liver endothelium lost its fenestrae. The defenestration remained throughout the 20 weeks of CD-HFD and was not affected by exercise (Fig. 11a). After 12 weeks of CD-HFD, the phosphorylation of endothelial nitric oxide synthase (eNOS)

decreased as did the expression of platelet-endothelial cell adhesion molecule-1 (PECAM-1) (Fig. 11b). Exercise did not affect the phosphorylation state of eNOS and did not restore the expression of PECAM-1 (Fig. 11c).

DISCUSSION

In the present study, we explored the selective benefits of physical activity to change the outcome of NAFLD triggered by a high-fat diet. We report that daily exercise alone halts the transition from NAFL to NASH, reduces steatosis, impedes the progression of fibrosis and reduces the incidence of tumor formation.

Previous studies evaluating the effect of lifestyle interventions have relied on post-intervention liver biopsy to gauge the improvement in histologic features of NASH [7, 21-23]. However, because dietary interventions designed for weight loss were inextricably linked, direct evidence that the effect on the liver was mediated by exercise could not be claimed. Conversely, our study design has navigated this obstacle and we find that exercise without dietary intervention impedes ballooning, a hallmark of NASH, and arrests the fibrotic progression of disease. Since these beneficial effects of an imposed exercise regimen occurred without concomitant weight loss and despite maintaining a high fat diet, we claim that exercise is beneficial despite the continued burden of overnutrition.

The experimental model we selected was a non-obesogenic, nutrient-deficient, high fat diet model, which offers the advantages of a rapid

onset of disease and mirrors the complexity of clinical NASH with fibrosis and development of tumors [24-25]. Hence it was a robust choice in which to test the short-term effects of regular exercise. In addition, the choline-deficiency presents the added liability of a disturbance in the phosphatidylcholine content of hepatic membranes including mitochondrial membranes, which is a source of mitochondrial dysfunction [26] and contributes to the pathogenesis of NAFLD in this experimental model.

Exercise lowered hepatic levels of triglycerides in our CD-HFD mice (Fig. 2, Fig. 5a), which is in line with non-invasive data collected from clinical studies [27]. Although the triggers of steatosis are multi-factorial in NAFLD, we can exclude increased de novo lipogenesis as feature of the CD-HFD model [28]. Lipogenic enzymes were downregulated in the NAFL mice and the phosphorylation status of HSL favoured lipolysis (Fig. 5d). Exercise interceded in this process by further suppressing activated ATPCL in addition to reducing the inhibition of ASL (Fig. 5d). The CD-HFD changed the uptake processes of fatty acids into the liver by upregulating CD36 and downregulating the solute carriers FATP2 and FATP5 and exercise did not modify these changes.

Exercise reduced the substantial increase of TNFα that was elicited by the CD-HFD (Fig. 6a), whereas the increased levels of TGFβ1 were not affected. Reduced levels of the proinflammatory TNFα have been linked to an amelioration of NAFLD, since anti-TNFα antibodies were reported to improve liver histology, to reduce circulating levels of aminotransferases and to deplete hepatic fat content [29]. Exercise-induced reduction in TNFα has also been linked to an improved insulin resistance [30]. Moreover, plasma levels of TNFα have been associated with the presence of ballooned hepatocytes [31]. Thus, our findings that the reduction of TNFα was linked to the absence of ballooning (Fig. 2b) and a lower NAFLD activity score (Fig. 2c) and lower ALT and AST concentrations (Fig. 4) are in keeping with previous studies. A novel biomarker purported be negatively correlated with the extent of NAFLD and, in particular. the degree of ballooning is the IGF-2 [31]. However, in our model, IGF-2 was highest in the sedentary 20 weeks CD-HFD group linked to the highest NAFLD score and was significantly reduced in the exercised group (Fig. 6c). Consequently, IGF-2 was positively rather than negatively correlated with disease in our model. In fact, IGF-2 was reported to increase in tumor bearing livers of mice subjected to a choline deficient diet and CCl4 treatment [32]. For this reason, the

reduced levels of IGF-2 produced in the exercise group likely reflect the fewer numbers of nodules formed.

The ability of exercise to prevent the histological progression of fibrosis (Fig. 3a) was confirmed by the changes in circulating markers of hepatic fibrosis, namely Pro-C3, Pro-C4 and C6M (Fig. 3c). In fact, the histological assessment of fibrosis correlated well with these circulating markers [33]. Pro-C3, a defined epitope of the NH2-terminal pro-peptide of type III procollagen, is a marker of active fibrogenesis and it is released by the protease ADAMTS-2 during collagen maturation, which is a prerequisite of efficient incorporation of collagen type III in collagen fibrils [34]. Pro-C4, a marker of collagen type IV formation reflects pericellular fibrosis and not bridging reticular fibrotic bands as does Pro-C3 [35]. C6M detects an internal epitope in the collagen type VI that is exposed by multiple matrix metalloproteinases when the collagen structure is degraded [36]. It is severely upregulated in the fibrotic space of Disse and portal tract stroma and engages in signaling related to the metabolic syndrome and fibrogenesis [36]. These fibrosis biomarkers were all significantly decreased in exercised mice compared to sedentary mice (Fig. 3c). In fact, the plasma concentrations of all three biomarkers

in the 20 weeks CD-HFD exercised group were comparable to those of the 12 weeks NAFL group.

This study confirms that exercise decreases the development of NASH-related liver cancer. Both the number of animals bearing tumor and that the number of tumors per liver were significantly reduced (Fig. 9a, 9b). We previously reported that regular physical activity decreases from 100% to 70% the occurrence of tumors in a model of NASH induced by the ablation of hepatocellular PTEN. We also confirmed that the exercise-related mechanism was activation of AMPK and inhibition of the mTOR/S6K pathway [13]. This mechanism holds for the current model of dietary-induced NASH. Exercise increased the activating phosphorylation of AMPK, resulting downstream in less phosphorylation of S6 (Fig. 9d).

In both genetic and dietary models of obesity, autophagy is suppressed in the liver, at least in part due to a reduction in the expression level of key autophagy molecules, such as Atg7 [37-38]. Autophagy deficiency is accompanied by defective insulin signaling and elevated ER stress. In our dietary model of NASH, the ER stress appeared to be chronic, as reflected by the absence of XBP1 expression and the induction of the pro-apoptotic transcription factor CHOP that transactivates pro-apoptotic

proteins (Fig. 7). As expected, evidence of an activated pro-apoptotic pathway was detected since Bax was upregulated and the ratio of Bax to Bcl2 was significantly elevated (Fig. 7). However, exercise has relieved the autophagic block that can accompany ER stress. Autophagy appeared to be activated by exercise as shown by the tendency for mTOR downregulation, an increase in LC3BII/LC3BI and an increase in mitochondrial recruitment of phosphorylated PINK indicative of mitophagy (Fig. 8). The benefits of an activated mitophagy process derives from the need to clear hepatocytes of dysfunctional mitochondria that accumulate in NASH [39-40]. Indeed, mitochondria in our CD-HFD model were compromised (Fig. 10). The cytochrome c control factor was significantly increased in all respirometry runs likely because of activation of the pro-apoptotic pathway and loss of cytochrome c and mitochondrial uncoupling (Fig. 10). In addition, decrease in maximal respiration and complex IV activity could be explained by a change in the expression of at least one component of complex IV (Fig. 10). Overall, our findings support the general view that exercise stimulates selective autophagic processes in the liver to alleviate hepatocytes of its deleterious burden of lipid overload and dysfunctional mitochondria [41].

LSECs undergo morphological and functional changes during steatosis [42]. One of the most remarkable phenotypic changes is defenestration or sinusoidal capillarization that appeared early in our model at the 12 weeks NAFL stage and before NASH and has been reported in other models of NASH [43]. During NALFD progression, LSECs acquire a pro-inflammatory phenotype and become effectors of liver inflammation in NASH and also promote fibrosis [44]. Exercise did not reverse this morphological change.

In conclusion, our work complements the small number studies that have evaluated the positive effect of lifestyle interventions on the histological features of NASH [21-23]. In addition, our work confirms in yet another model that exercise not only arrests the development of liver cancer [13] but attenuates its progression [45]. Finally, we provide direct evidence that exercise alone can be a therapeutic measure and not only a preventive measure in NAFLD and this should offer hope to patients who fail at sustained, consequential dietary changes [46].

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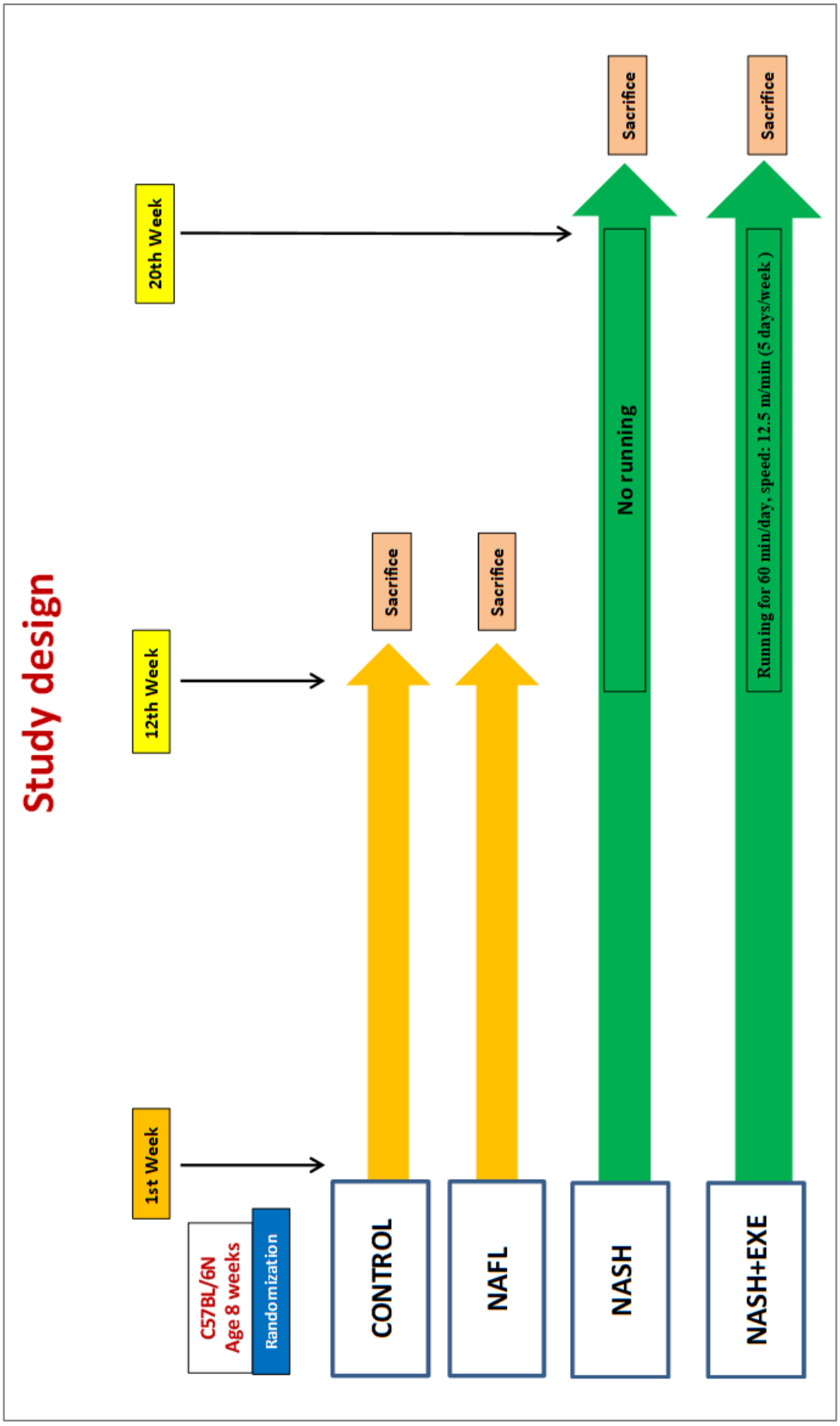
Figures

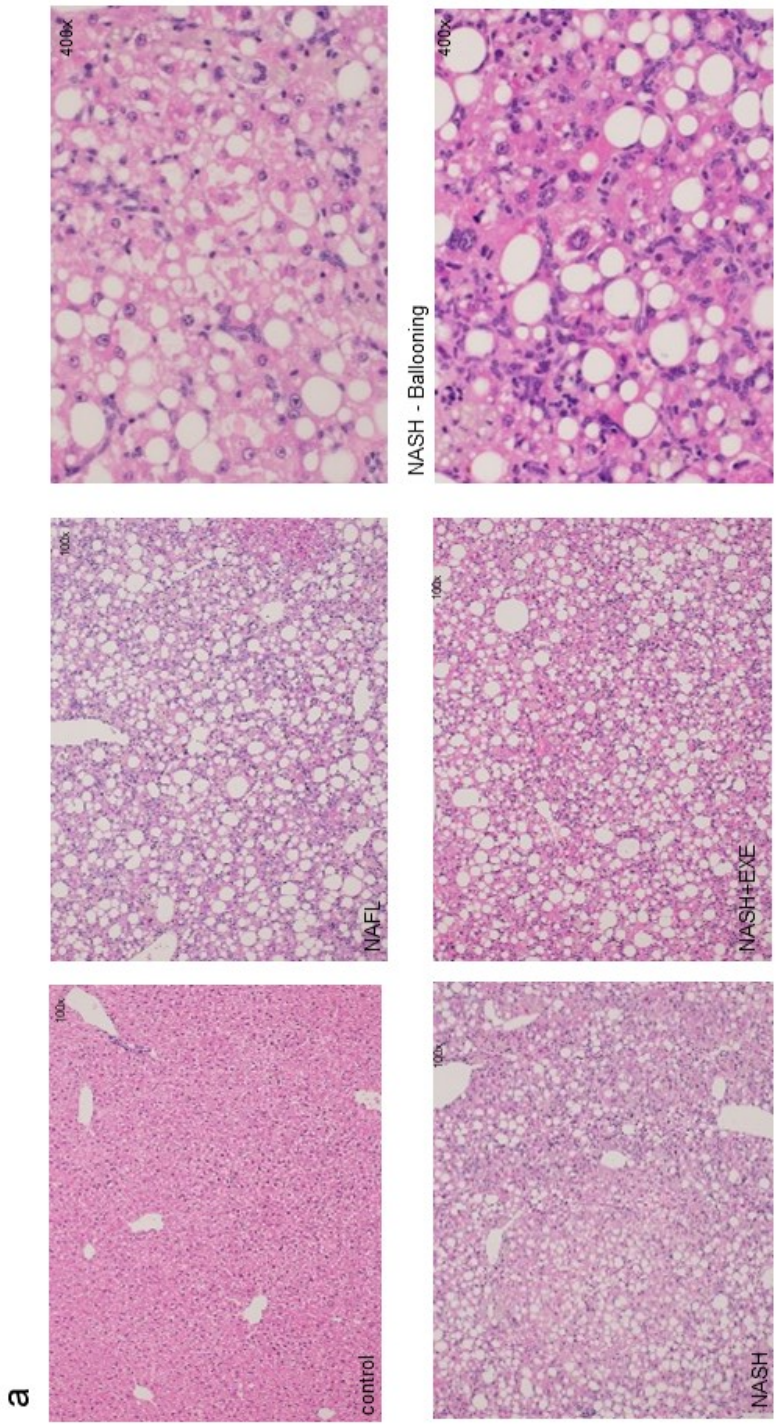
Figure 1. Schematic outline of the study design



Schematic outline of the study design

Male C57Bl/6N mice were randomized to one of four groups: 1) the control group (*n*=11) was fed a standard diet and tissues were collected after 12 weeks; 2) the non-alcoholic fatty liver (NAFL) group (*n*=11) was fed a choline-deficient high fat (CD-HFD) diet for 12 weeks before tissue collection; 3) the non-alcoholic steatohepatitis (NASH) group (*n*=11) was fed a CD-HFD for 20 weeks and remained sedentary before tissue collection; 4) the NASH + exercise (EXE) group (*n*=11) was fed a CD-HFD for 20 weeks but with treadmill running at 12.5 m/min imposed from weeks 12 to 20.

Figure 2. Effect of exercise on liver histology in mice fed a choline deficient-high fat diet (CD-HFD)



b

	Ballooning score		Total
	0-1	*2*	
NASH_Sed	1	7	8
NASH_Exe	9	2	11

c

		NAFLD Activity Score			Total
	0-5	*6*	*7*	*8*	
NAFL	0	9	0	0	9
NASH_Sed	0	0	1	7	8
NASH_Exe	0	9	2	0	11

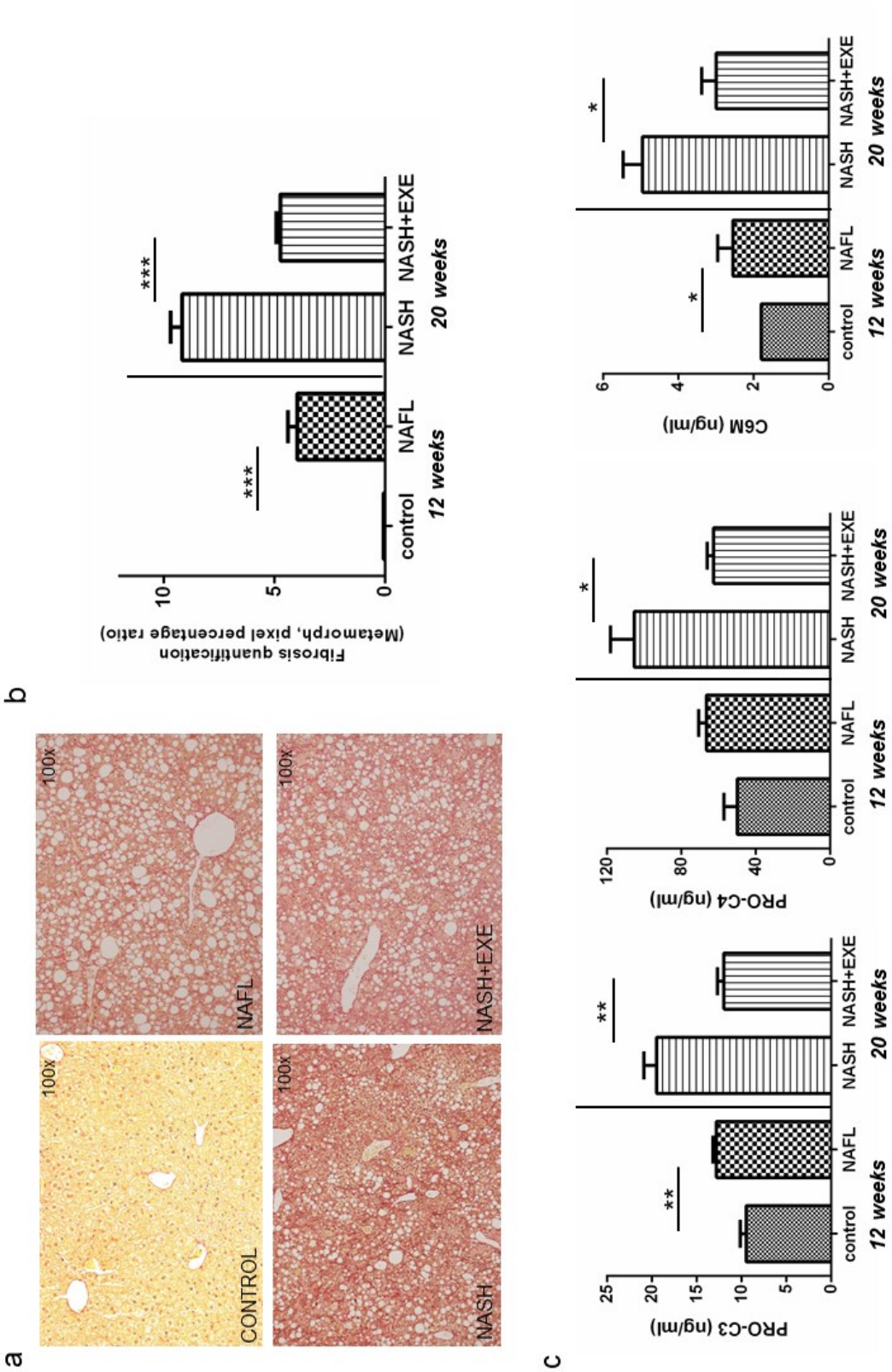
Effect of exercise on liver histology in mice fed a choline deficient-high fat diet (CD-HFD)

a) Microscopy of hematoxylin and eosin (H&E) - stained liver sections showing steatosis in the NAFL, NASH and NASH+exercise (EXE) groups (magnification x100) and the presence of ballooning in hepatocytes only in the NASH group (magnification x400).

b) Frequency table showing the ballooning score in the NASH sedentary (NASH_Sed) and NASH+EXE groups. The ballooning was significantly lower in the NASH+EXE group (Fisher's exact test, p=0.005).

c) Frequency table showing the NAFLD activity score in the NAFL, NASH sedentary and NASH+EXE groups. The score was significantly lower in NASH+EXE than in the NASH sedentary group (Fisher's exact test with Freeman-Halton extension, $p < 0.0001$).

Figure 3. Effect of exercise on liver fibrosis



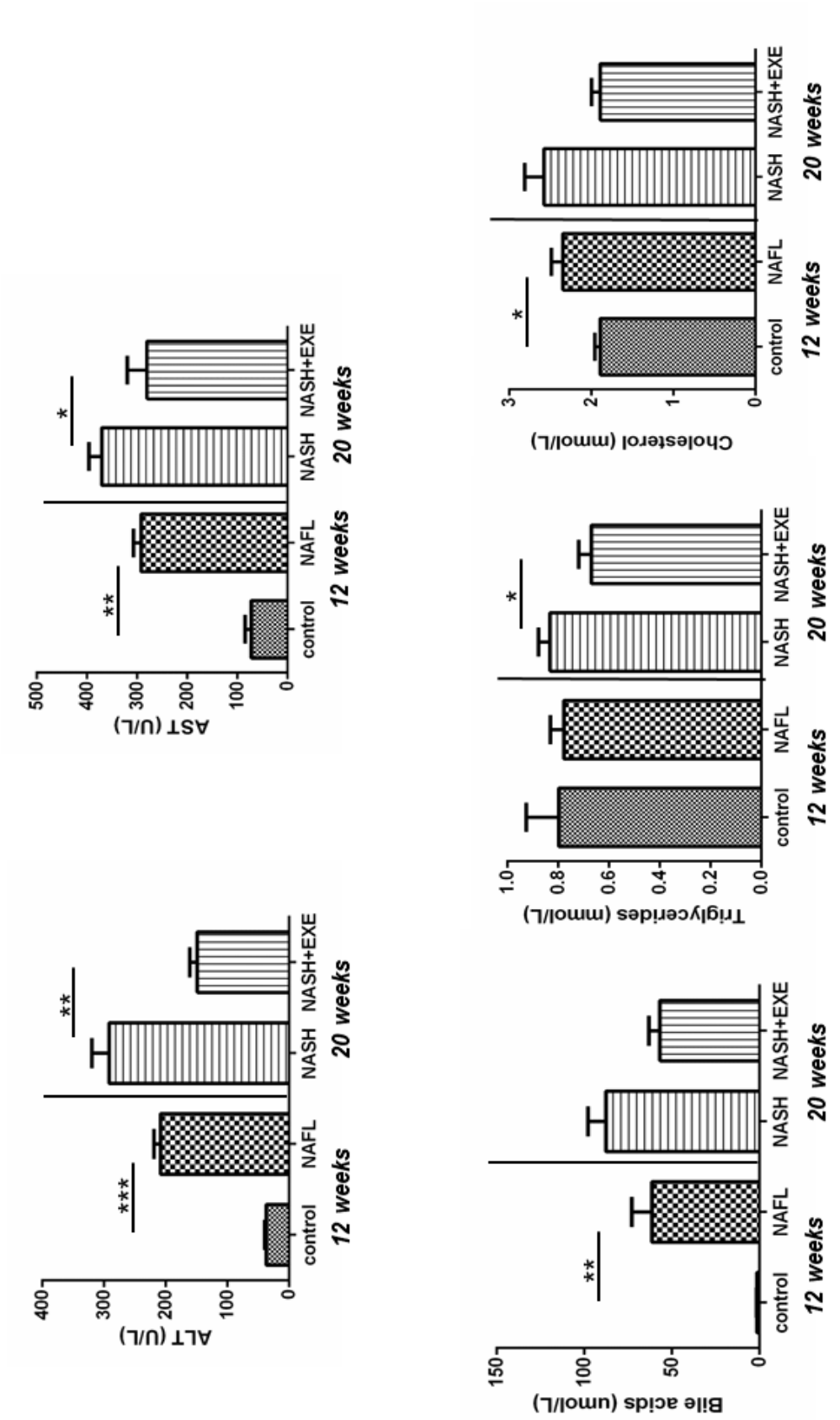
Effect of exercise on liver fibrosis

a) Bright-field microscopy of Sirius Red-stained liver sections from C57BL/6N mice fed a control diet or a choline deficient–high fat diet (CD-HFD). Fibrosis was absent in controls. Fibrosis was present in the NAFL and NASH+exercise (EXE) groups but was highest in the NASH sedentary group (magnification x100).

b) Quantification of fibrosis shown in panel **a**. Images were quantified with the MetaMorph® analysis software. Fibrosis was higher in NAFL than in control groups and higher in NASH sedentary than in NASH+EXE group ($p < 0.0001$).

c) Fibrosis biomarkers in plasma. PRO-C3, PRO-C4 and C6M concentrations were measured in the plasma of mice from the control, NAFL, NASH and NASH+EXE groups. PRO-C3 and C6M were significantly higher in NAFL than in controls and in NASH sedentary than in NASH+EXE ($p < 0.05$). C6M was significantly higher in NASH sedentary than in NASH+EXE *(*$p < 0.05$). *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$

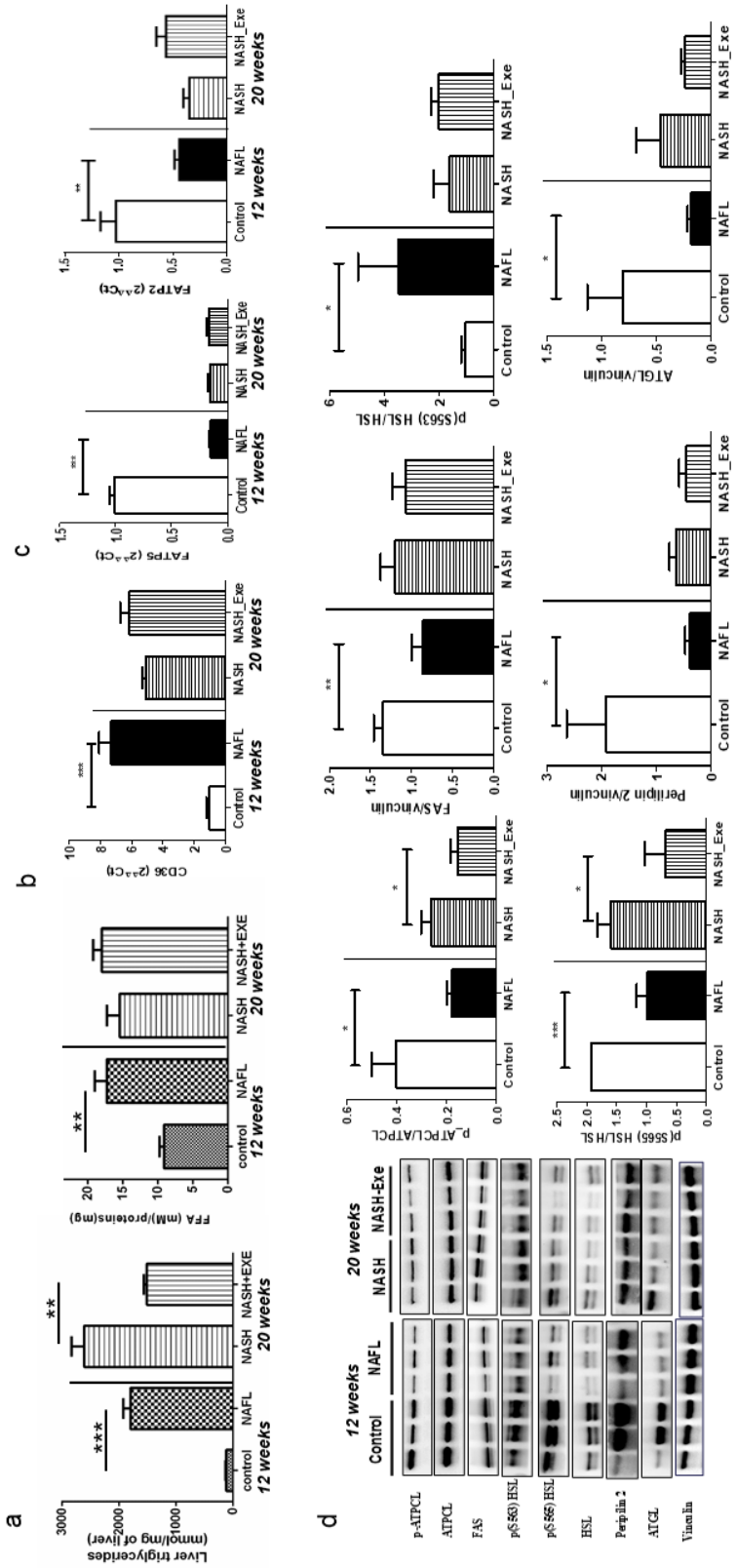
Figure 4. Effect of exercise on plasma biochemistry



Effect of exercise on plasma biochemistry

Plasma concentrations of alanine transaminase (ALT), aspartate transaminase (AST), total bile acids, triglycerides and cholesterol were compared in mice fed a control diet or a choline deficient – high fat diet (CD-HFD) for 12 weeks (NAFL), and in mice fed a CD-HFD for 20 weeks with (NASH+EXE) or without exercise (NASH). ALT and AST were higher in NAFL than in controls, and higher in NASH sedentary than in NASH+EXE ($p < 0.05$). Bile acids were elevated in NAFL *vs* controls ($p < 0.001$). Triglycerides were lower in NASH+EXE than in NASH sedentary ($p<0.05$). Cholesterol was higher in NAFL than in controls ($p<0.05$). *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$

Figure 5. Effect of exercise on hepatic lipid metabolism



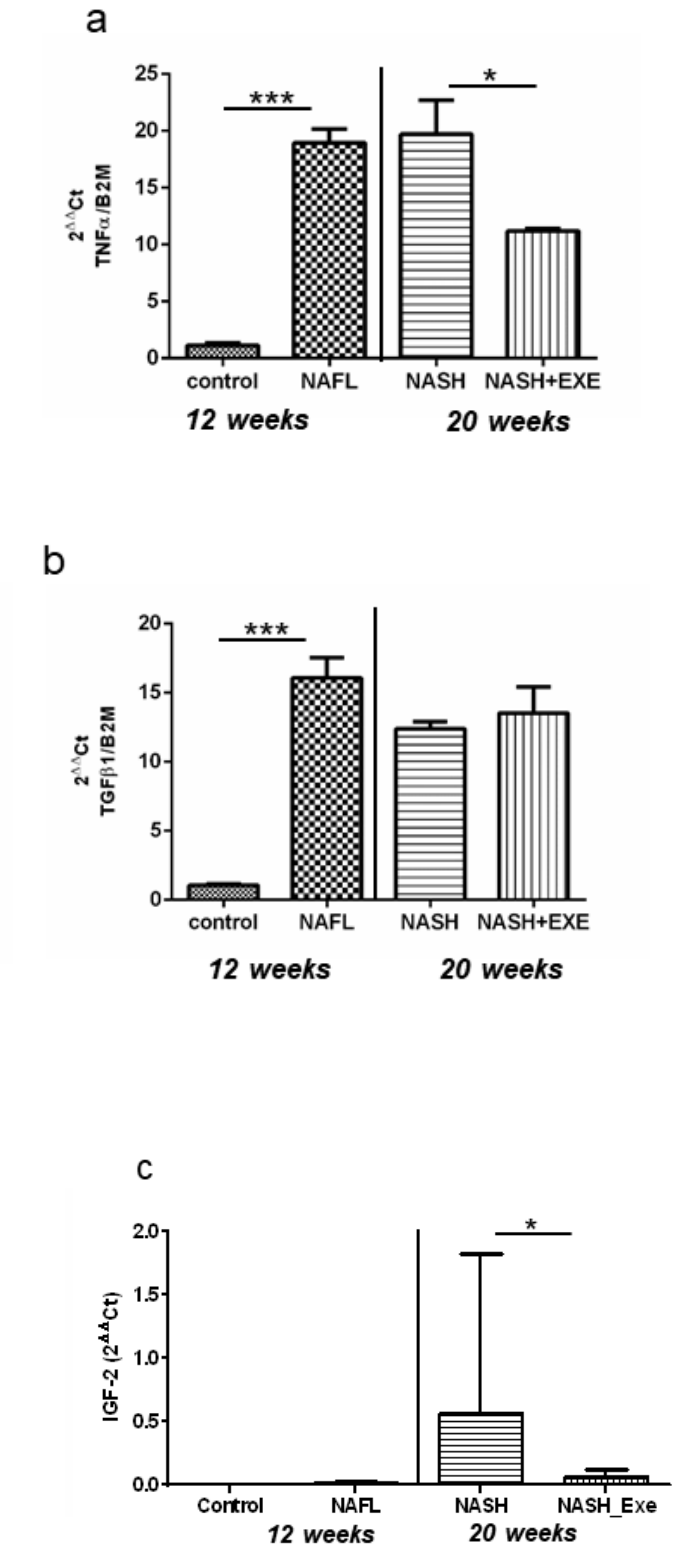
Effect of exercise on hepatic lipid metabolism

a) Plasma concentrations of triglycerides and free fatty acids (FFA) were compared in mice fed a control diet or a choline deficient – high fat diet (CD-HFD) for 12 weeks (NAFL), and in mice fed a CD-HFD for 20 weeks with (NASH+EXE) or without exercise (NASH).

b) Semi-quantitative PCR measurement of CD36 mRNA levels in liver extracts relative to β2-microglobulin. CD36 was higher in NAFL than in controls ($p < 0.001$). **c)** Semi-quantitative PCR measurements of fatty acid transport protein 2 (FATP2) and fatty acid transport protein 5 (FATP5) in liver extracts relative to β2-microglobulin. FATP5 and FATP2 were lower in NAFL than in controls (n=6 per group).

d) Immunoblots of liver homogenates comparing control and NAFL mice, and NASH and NASH+EXE groups. Immunoblots were quantified and normalized with vinculin and are reported as mean ± SD (n=3 per group). *HSL, hormone-sensitive lipase; ATGL, adipose triglyceride lipase; ATPCL, ATP citrate lyase; FAS, fatty acid synthase; HSL, hormone sensitive lipase.* *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$

Figure 6. Effect of exercise on expression of liver biomarkers



Effect of exercise on expression of liver biomarkers

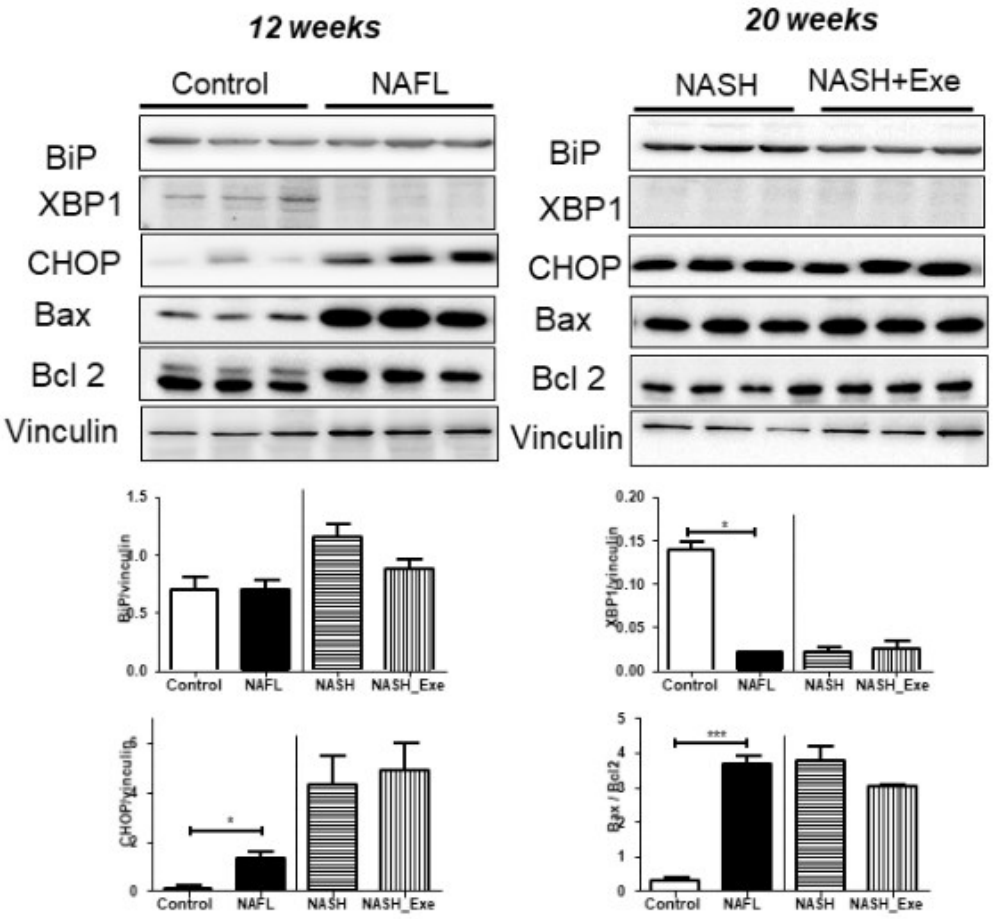
Semi-quantitative PCR analysis of mRNA expression relative to β2 microglobulin in livers from control, NAFL, NASH and NASH+EXE groups.

a) Tumor necrosis factor α (TNFα) was increased in NAFL vs control ($p<0.001$) and NASH+EXE vs NASH sedentary ($p=0.05$).

b) Transforming growth factor β (TGFβ) was increased in NAFL vs control group ($p<0.001$) but not changed in NASH vs NASH+EXE.

c) Insulin-like growth factor 2 (IGF-2) was increased in the NASH sedentary relative to NASH+EXE. Groups are described in Fig.1. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$

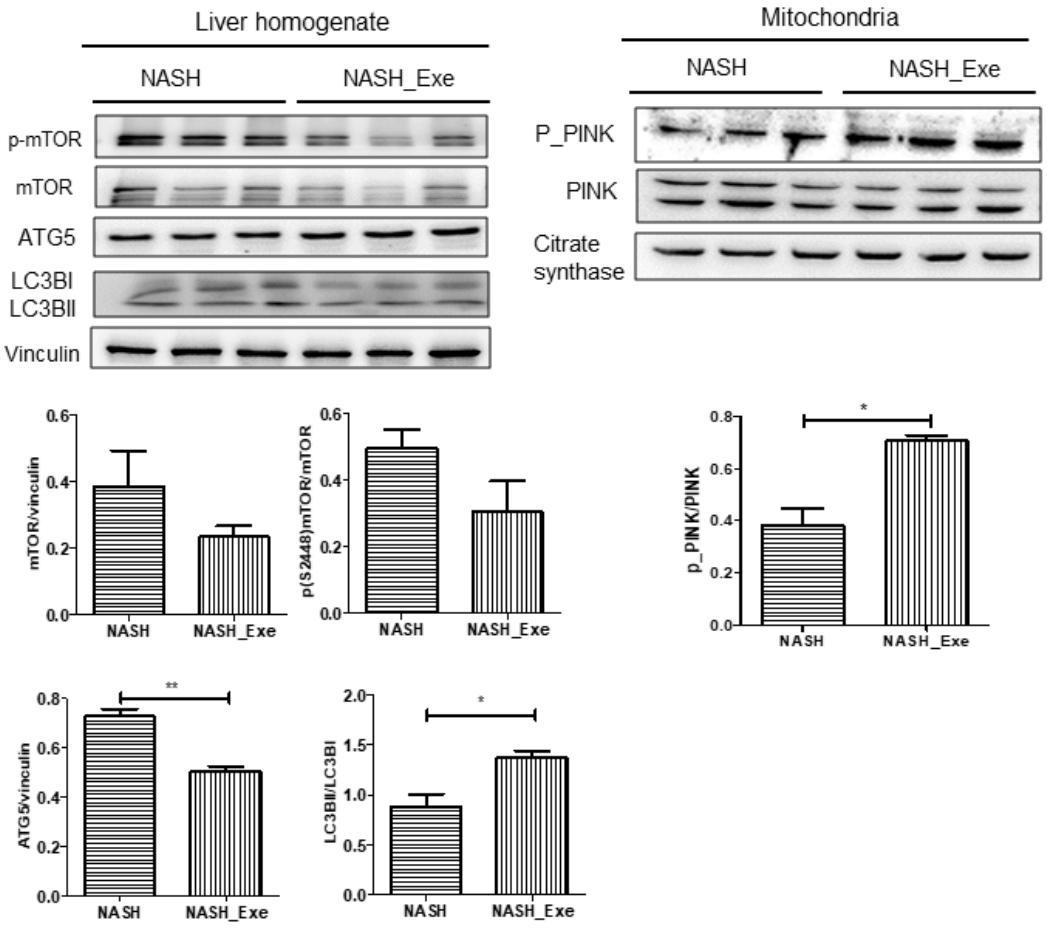
Figure 7. Effect of diet and exercise on ER stress and apoptosis



Effect of diet and exercise on ER stress and apoptosis

Immunoblots (upper panel) and quantification (lower panel) of proteins expressed in livers from control, NAFL, NASH and NASH+EXE groups. Binding immunoglobulin protein (BiP) was not different between groups. X-box binding protein (XBP-1) was lower in the NAFL and both NASH groups ($p < 0.05$). C/EBP homologous protein (CHOP) expression was higher in NAFL and NASH groups than in controls. The ratio of Bcl-2 associated X protein (BAX) to Bcl-2 was elevated in NAFL vs control but was not changed by exercise. Vinculin served as the loading control. Groups are described in Fig.1. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$

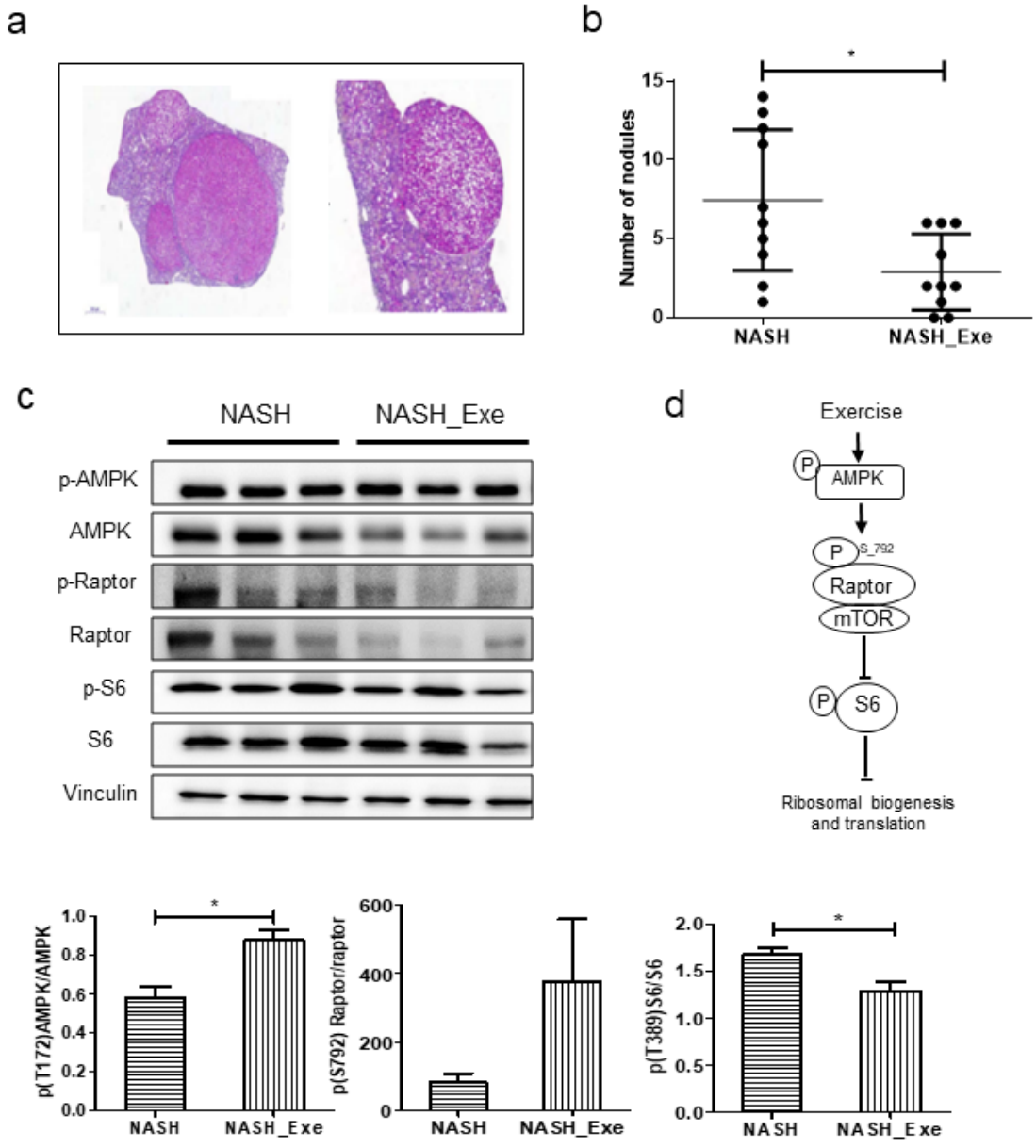
Figure 8. Effect of exercise on autophagy



Effect of exercise on autophagy

Immunoblots of protein expression in liver homogenates (left panel) and mitochondria (right panel) for markers of autophagy in the NASH sedentary and NASH+EXE groups. mTOR and its phosphorylation tended to decrease in the NASH+EXE group. Autophagy related gene 5 (ATG5) tended to decrease in the NASH+EXE group. The ratio of Light Chain 3BII (LCBII) to LCBI was increased in the NASH+EXE group ($p < 0.05$). Vinculin served as the loading control. The phosphorylation of PTEN-induced kinase (P-PINK) increased in mitochondria of the NASH+EXE group ($p<0.05$). Citrate synthase served as the loading control. Groups are described in Fig.1. *, $p < 0.05$; **, $p < 0.005$

Figure 9. Effect of exercise on liver carcinogenesis

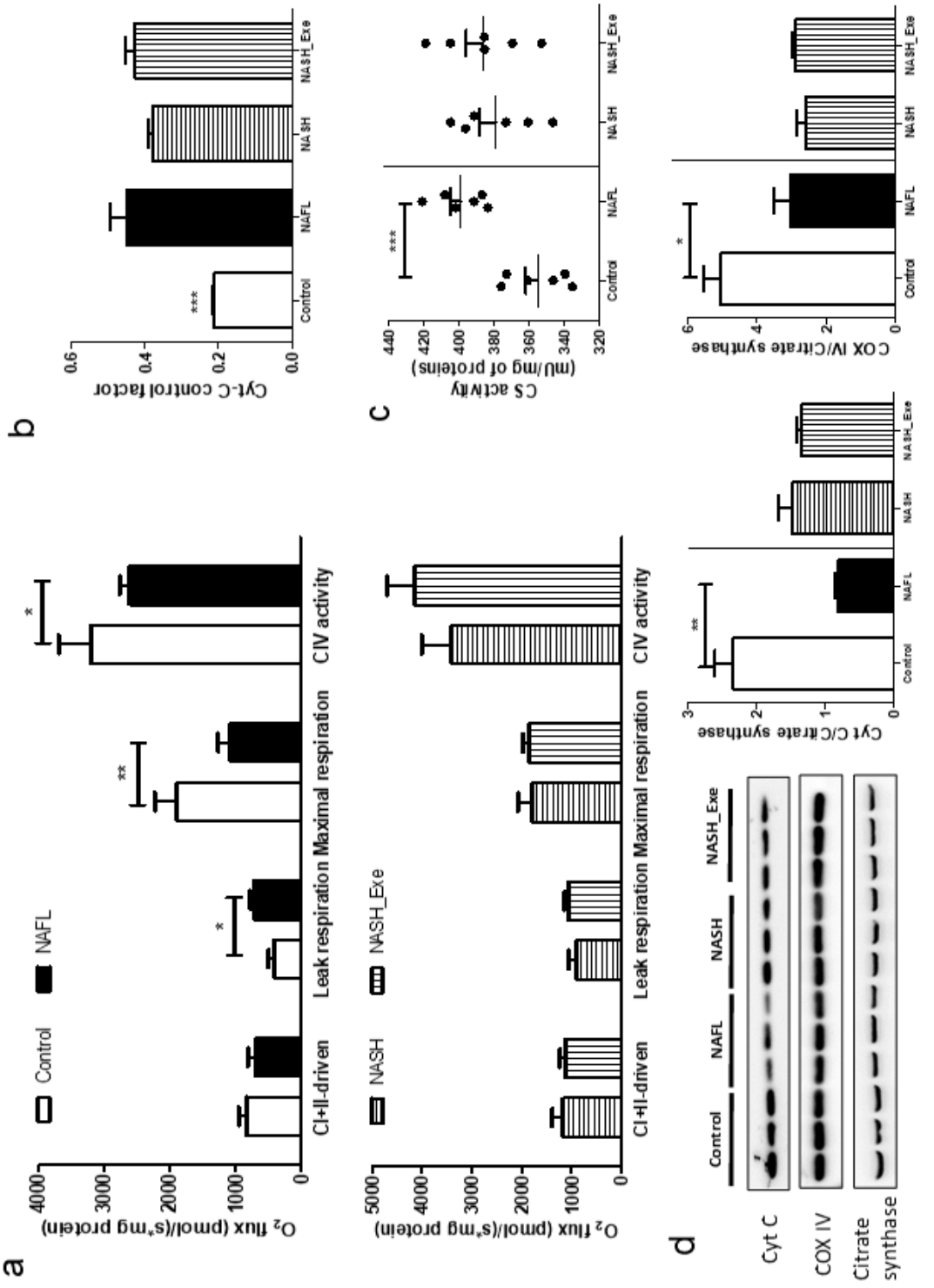


Effect of exercise on liver carcinogenesis

a) Representative bright-field images of hematoxylin and eosin (H&E) - stained liver sections with nodules in NASH mice (magnification x40). **b)** Quantification of the number of liver nodules in NASH mice. NASH+EXE mice developed fewer nodules than did NASH sedentary mice ($p < 0.05$).

c) Immunoblots for protein expression and phosphorylation status of AMP-activated protein kinase (AMPK), regulated associated protein of mTOR (Raptor) and ribosomal protein S6 were quantified and normalized for vinculin. Phosphorylation of AMPK was increased and phosphorylation of S6 was decreased in the NASH-EXE group ($p<0.05$). **d)** Schematic representation of the AMPK – Raptor – S6 cascade. Groups are described in Fig.1. *, $p < 0.05$

Figure 10. Effect of exercise on mitochondrial bioenergetics



Effect of exercise on mitochondrial bioenergetics

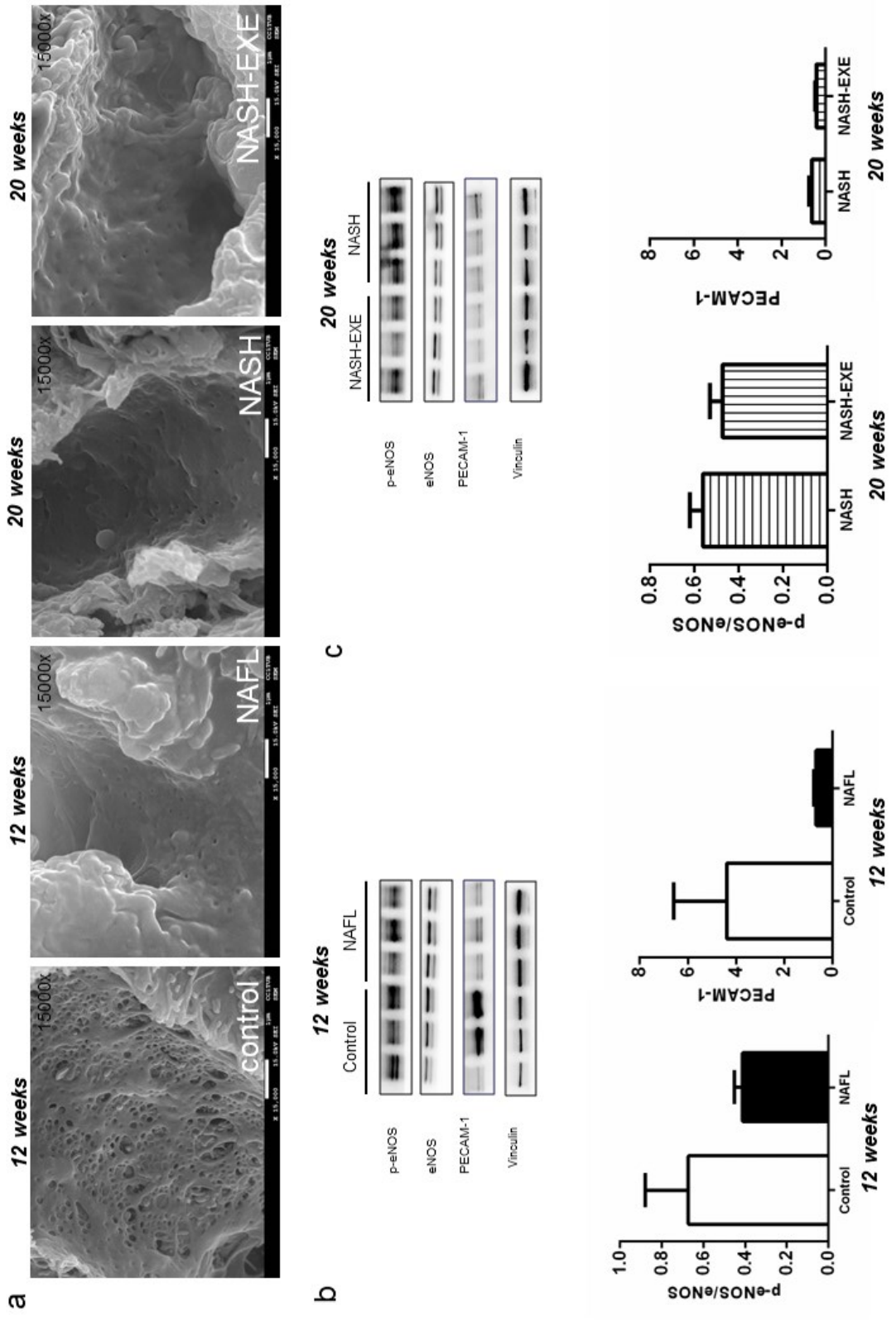
a) High-resolution respirometry of oxygen consumption rates (OCR) in mitochondria isolated from livers of control *vs* NAFL group (12 weeks treatment) (upper panel) and NASH sedentary *vs* NASH+EXE groups (bottom panel). OCRs were measured with a O2k Oroboros instrument. Mitochondria were exposed to sequential additions of pyruvate/malate, glutamate, succinate, ADP, cytochrome c, oligomycin, rotenone, antimycin A, TMPD, ascorbate and azide. Coupled complex I and complex II–driven, leak respiration, maximal respiration and complex IV OCRs were recorded and normalized for protein (n = 3 per group).

b) Comparison of cytochrome *c* control factors. OCRs were measured as the fractional change of flux after addition of excess cytochrome *c* and calculated as (Flux CI+II_{cytc} - Flux CI+II)/ Flux CI+II_{cytc}. The ratio significantly elevated in NAFL and NASH groups relative to control (p<0.05).

c) Comparison of mitochondrial citrate synthase activity in control *vs* NAFL group and NASH sedentary *vs* NASH+EXE groups. Activity was normalized for mitochondrial protein and was higher in NAFL *vs* control ($p < 0.01$).

d) Immunoblot showing expression of cytochrome c and cytochrome c oxidase IV (COXIV) in mitochondria. Cytochrome c and COXIV were lower in NAFL *vs* control groups ($p < 0.05$). *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$

Figure 11. Effect of exercise on liver endothelium



Effect of exercise on liver endothelium

a) Scanning electron microscopy of endothelial cells from livers of control and CD-HFD mice (magnification x15000). The NAFL, NASH and NASH+EXE groups are as described in Fig. 1.

b) Immunoblots of proteins expressed in liver homogenates of control and NAFL groups (12 weeks treatment).

c) Immunoblots of proteins expressed in liver of NASH and NASH+EXE groups (20 weeks treatment). Immunoblots were quantified and normalized with vinculin. eNOS, endothelial nitric oxide synthase; PECAM, platelet endothelial cell adhesion molecule. The NAFL, NASH, and NASH+EXE groups are as described in Fig. 1.