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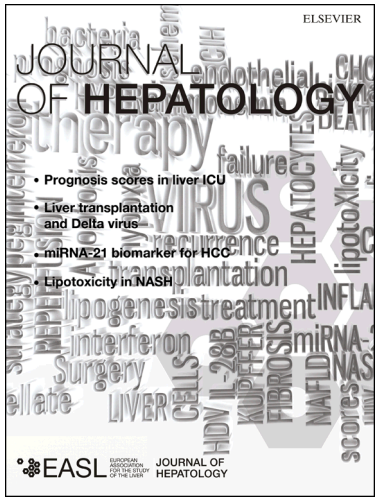

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Regular exercise decreases liver tumors development in hepatocyte-specific PTEN-deficient mice independently of steatosis

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HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PTEN, phosphatase and tensin homolog deleted from chromosome 10; NAS, NAFLD activity score; qPCR, Real-Time Quantitative Polymerase Chain Reaction; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; Ddit4, DNA-damage-inducible transcript 4; ACC, Acetyl-CoA carboxylase; FAS, Fatty acid synthase

Keywords:

Non-alcoholic fatty liver disease; non-alcoholic steatohepatitis; hepatocellular carcinoma; AMPK; mTOR.

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Authors contributions:

Anne-Christine Piguet: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; statistical analysis

Uttara Saran: acquisition of data; analysis and interpretation of data; statistical analysis

Cedric Simillion: analysis and interpretation of data; statistical analysis

Irene Keller: analysis and interpretation of data; statistical analysis

Luigi Terracciano: analysis and interpretation of data; critical revision of the manuscript for important intellectual content

Helen L. Reeves: analysis and interpretation of data; critical revision of the manuscript for important intellectual content

Jean-François Dufour: study concept and design; analysis and interpretation of data; critical revision of the manuscript for important intellectual content; obtained funding; study supervision

ABSTRACT

Background & Aims: Unhealthy lifestyles predispose to non-alcoholic steatohepatitis (NASH), which may further result in the development of hepatocellular carcinoma (HCC). Although NASH patients benefit from physical activity, it is unknown whether regular exercise reduces the risk of developing HCC. Therefore, we studied the effect of regular exercise on the development of HCC in male hepatocyte-specific PTEN-deficient mice (*AlbCrePten*$^{flox/flox}$), which develop steatohepatitis and HCC spontaneously.

Methods: Mice were fed a standardized 10% fat diet and were randomly divided into exercise or sedentary groups. The exercise group ran on a motorized treadmill for 60 minutes/day, 5 days/week during 32 weeks.

Results: After 32 weeks of regular exercise, 71% of exercised mice developed nodules larger than 15 mm^3 vs 100% of mice in the sedentary group. The mean number of tumors per liver was reduced by exercise, as well as the total tumoral volume per liver. Exercise did not affect steatosis and had no effect on the Non-alcoholic fatty liver disease (NAFLD) Activity Score (NAS). Exercise decreased tumor cell proliferation. Mechanistically, exercise stimulated the phosphorylation of AMPK and its substrate raptor, which decreased the kinase activity of mTOR.

Conclusions: These data show a benefit of regular exercise on the development of HCC in an experimental model of NASH and offer a rationale for encouraging predisposed patients to increase their physical activity for the prevention of HCC.

Word count: 224 words

Keywords: Non-alcoholic fatty liver disease; non-alcoholic steatohepatitis; hepatocellular carcinoma; AMPK; mTOR.

INTRODUCTION

With more than half a million new cases diagnosed each year in the world and with a similar number of deaths, liver cancer is the fifth most commonly diagnosed cancer in the world and the second most common cause of cancer-related mortality [1]. Hepatocellular carcinoma (HCC) represents the major primary malignancy of the liver [2], with an incidence rate that is growing in the Western World [3] . The main reasons for this increase are the epidemic of hepatitis C virus, alcohol abuse, and the surge in obesity [1-3]. Moreover, growing evidence supports the role of non-alcoholic fatty liver disease (NAFLD) and its complication, non-alcoholic steatohepatitis (NASH), as risk factors for HCC [4]. NAFLD, characterized by excessive fat accumulation in the liver, termed steatosis, is the most common liver disease in developed countries. The disease can progress to NASH with the appearance of histologic features of hepatocellular inflammation, ballooning, Mallory-Denk bodies and fibrosis. NAFLD is strongly associated with the prevalence of obesity and type 2 diabetes [5], and both diseases are established as major risk factors for the development of HCC [6, 7] . Lifestyle changes, which include weight loss and increasing physical activity, are the best preventive and curative measures against obesity and diabetes, and several studies have demonstrated the beneficial effect of physical activity in the prevention of the progression of NAFLD to NASH [8].

Accumulating evidence suggests that physical activity and regular exercise provide other health benefits, including relief of cancer treatment-related symptoms, such as fatigue [9], but also protection against cancer and improved survival in those with cancer. It has recently been demonstrated that physical inactivity is associated with 5% to 12% of breast and colon cancers [10], while for breast cancer sufferers, physical activity has been shown to be associated with decreased incidence and reduced risk for recurrence and mortality [11, 12]. Similarly, a reduced risk and

improvement of survival were observed with physical activity in patients with colorectal cancer [12-14]. Literature concerning physical activity and HCC is sparse, although one study has reported a reduced hazard ratio for HCC development with low level of activity, with a further decreased risk of tumor development demonstrated with higher physical activity [15].

Using the hepatocyte-specific PTEN (phosphatase and tensin homolg deleted from chromosome 10) -deficient mouse model (*AlbCrePten*$^{flox/flox}$), which is characterized by the spontaneous development of steatohepatitis and HCC [16], we aimed in the present study to verify whether regular exercise may impact liver tumor growth in a fatty liver environment.

MATERIAL AND METHODS

Animals and dietary treatment

Male *AlbCrePten*$^{flox/flox}$ mice [16] were supplied from our own animal facility (University of Berne, Berne, Switzerland). The mice were 7–9 weeks old and were divided into two groups: sedentary (n=10) and exercise (n=10).

All mice received a standard diet (Kliba Nafag, Kaiseraugst, Switzerland, the diet composition is presented in Supplementary Table S1). The mice had free access to both food and distilled water throughout the duration of the experiments, except for duration of their experimental exercise, and that of the glucose tolerance test and blood sampling, which required animals to be fasted. Body mass was measured weekly. Animals received humane care in accordance with the regulations for laboratory animals and experiments were performed following protocols approved by the animal use committee of the Canton of Berne, Switzerland.

Exercise protocol

Mice were given an exercise regime over 32 weeks. At the beginning of the experiment, mice of the exercise group were gradually introduced to running on a treadmill (Förderband GFB, Elmotec, Kleindöttingen, Switzerland) by exposing them to increasing speed of the treadmill for increasing amounts of exercise time (acclimatisation phase; Supplementary Fig. 1). After 5 weeks of acclimatisation, the animals exercised during their light phase for 60 minutes/day, 5 days/week at running speed of 12.5 m/minutes. Mice attempting to rest were encouraged to move by gently tapping on their tail and their back. Sedentary mice were kept in their cages. Both exercised and sedentary mice were sacrificed 72 hours after the final exercise session.

A second set of experiments was performed, where mice (n=3) were exposed to a single bout of exercise on the treadmill for 60 minutes at 12.5 m/minutes. The sedentary control group (n=3) were exposed to the treadmill for 1 hour, but were to remain motionless. Both groups of mice were sacrificed 15 minutes after treadmill exposure i.e. at 1 hour and 15 minutes. Mice of both groups had no access to food during these exposures.

Further methods are described in Supplementary Material.

RESULTS

Effect of exercise on the development of tumoral nodules

The incidence of tumoral nodule development in *AlbCrePten*$^{flox/flox}$ mice was significantly reduced by exercise, with 71% of exercised mice developing liver nodules, compared to 100% of sedentary mice (Fig. 1A). As shown in Fig. 1A, the mean number of nodules larger than 15 mm^3 per liver was reduced by exercise (1.8±0.8 vs 2.8±2.3). In addition, measurement of the size of the nodules larger than 15 mm^3 and calculation of the total volume of nodules per liver showed that the combined volume in liver of exercised mice was less than half of that of mice in the sedentary group (444±551 vs 945±1007). The distribution between the total volume of tumoral nodules per liver and the number of mice (Fig. 1B) confirmed that a significantly higher number of sedentary *AlbCrePten*$^{flox/flox}$ mice developed larger nodules compared with the exercised mice.

These results indicated that regular exercise had beneficial effects on the development of liver tumors in *AlbCrePten*$^{flox/flox}$ mice.

Effects of exercise on metabolic and physiologic parameters

AlbCrePten$^{flox/flox}$ mice are characterized by insulin hypersensitivity and enhanced glucose clearance after oral glucose administration [16]. To assess whether glucose metabolism was affected by regular exercise in *AlbCrePten*$^{flox/flox}$ mice, a glucose tolerance test was performed in overnight fasted animals after 30 weeks of exercise. No differences in glucose level between exercise and sedentary animals were observed after glucose injection (Supplementary Fig. 2A). Similarly, basal fasted and fed glucose levels were not affected by exercise after 30 weeks of exercise (Supplementary Table 2).

There was a significant decrease in the body weight of mice after 32 weeks of regular exercise (p=0.03; Supplementary Fig. 2B). The weight gain between the first and the last week of exercise was also significantly reduced (p=0.005; Supplementary Fig. 2C). There was a trend (p=0.06) toward a reduction in the epididymal fat mass in regularly exercised mice (Supplementary Fig. 2D). In contrast, the liver weight and the liver weight per body weight ratio were not significantly affected after 32 weeks of exercise (Supplementary Fig. 2E-F).

These data showed that regular exercise significantly reduced the body mass of *AlbCrePten*$^{flox/flox}$ mice. This reduction could partly be explained by a reduction in the fat mass of these animals. In contrast, regular exercise did not affect the glucose level or the glucose metabolism in *AlbCrePten*$^{flox/flox}$ mice.

Effect of exercise on liver steatosis and hepatic injury

Several data demonstrated the beneficial effect of regular exercise on fatty liver severity [17, 18]. Steatosis and hepatic injury were therefore assessed by evaluating H&E-stained liver sections. Exercise did not affect steatosis (Fig. 2 and Supplementary Table 3). This was further confirmed by Oil Red O staining and by determination of the hepatic triglyceride content (Supplementary Fig. 3). A reduction in lobular inflammation was observed in the exercised mice, but the difference was not significant. No ballooned hepatocyte was observed in the liver of both exercise and sedentary animals. All these parameters resulted in a similar NAS between the two groups. These data showed that regular exercise did not affect liver injury induced by the loss of *Pten* expression in *AlbCrePten*$^{flox/flox}$ mice.

Effect of exercise on AMPK-mTOR signaling

As mice were sacrificed 72 hours after the last exercise session, a time period allowing signaling pathways affected by exercise to recover to their basal levels, the direct effect of exercise in liver tissue could not be assessed. Therefore a second set of experiments was performed with animals sacrificed immediately after a single bout of exercise. We hoped in this way to be able to understand the mechanism leading to the effect of exercise on tumor growth in *AlbCrePten*$^{flox/flox}$ mice.The effect of exercise on the AMPK (AMP-activated protein kinase) – mTOR (mammalian target of rapamycin) signaling pathway was assessed in the liver tissue, firstly by quantifying the phosphorylation of the α subunit of the AMP kinase, as a measure of AMP kinase activation [19]. The downstream phosphorylation of Raptor at Ser792, the targeted site of AMPK whose phosphorylation results in the inhibition of the complex mTOR-Raptor (also known as mTOR complex 1, mTORC1) [20], was also assessed, as was the phosphorylation of the S6 ribosomal protein as a measure of the activity of the complex mTOR-Raptor. A single bout of exercise resulted in an increase in the phosphorylation of AMPK and of Raptor and in a decrease of the phosphorylation of the S6 ribosomal protein in the liver tissue (Fig. 3A-C). Moreover the hepatic expression of *Ddit4* (DNA-damage-inducible transcript 4), which is involved in mTORC1 inhibition upon stresses, was also induced 15 minutes after the end of a single bout of exercise (Fig. 3D). Taken together, these data demonstrated the inhibition of the hepatic mTORC1 activity immediately after exercise in *AlbCrePten*$^{flox/flox}$ mice.

The mTORC1 complex, formed among others of mTOR and Raptor, is involved in cell growth and cell proliferation[21]. Therefore, cell proliferation was assessed by Ki67 immunohistostaining in nodules larger and smaller than 15 mm^3 observed in liver of *AlbCrePten*$^{flox/flox}$ mice. Cell proliferation in nodules larger than 15 mm^3 was significantly decreased by exercise ($p=0.036$) and showed a trend towards reduction

in nodules smaller than 15 mm^3 present in liver tissue (p=0.06; Fig. 4). The number of Ki67-positive cells in liver tissue was also significantly decreased by regular exercise compared with no exercise (data not shown). Taken together, these results suggested that regular exercise led to a decrease of hepatocellular cell proliferation in *AlbCrePten*$^{flox/flox}$ mice. This effect could partly been explained by the decrease of mTORC1 activity induced by the repetitive impact of exercise on AMPK activity and on *Ddit4* gene expression, leading to a decrease of tumor growth.

Effect of exercise on metabolism signaling

It is well known that exercise induces many beneficial metabolic effects. To understand the effect of exercise on metabolic pathways in *AlbCrePten*$^{flox/flox}$ mice, RNA-Seq analysis was performed on liver tissue of animals sacrificed immediately after a single bout of exercise or after sedentariness. The DESeq2 software was used to calculate differential expression between genes in the exercised group and the sedentary group. To detect which pathways are specifically affected by exercise, a gene set enrichment analysis (GSEA) was performed on the output of DESeq2 using the newly developed SetRank method (Supplementary Table 4). By applying more stringent parameters, 6 pathways were found to be significantly altered. Interestingly, these pathways were mostly involved in fatty acid metabolism (Fig. 5).

AMPK activation is known to phosphorylate and inactivate a number of metabolic enzymes involved in lipid metabolism, especially ACC, a key enzyme in fatty acid synthesis whose phosphorylation by AMPK leads to its enzymatic inactivation [19]. The phosphorylation of ACC at the AMPK target site Serine 79 was increased in the liver tissue *AlbCrePten*$^{flox/flox}$ mice immediately after the end of the exercise session (Supplementary Fig. 4), confirming among others the effect of exercise on AMPK activation. The inhibition of the enzymatic activity of ACC was accompanied by a

decrease in FAS expression, another key enzyme in fatty acid synthesis (Supplementary Fig. 4).

Taken together these data showed an inhibitory effect by exercise on lipogenesis immediately after the end of exercise. However, this was not translated over the long term into an effect on liver steatosis in our animal model, as shown above.

DISCUSSION

In the present study, regular exercise has been shown to have a beneficial negative impact on the development of HCC in an experimental model of NASH, characterized by the loss of Pten and the overactivation of mTOR. A reduction in the number and the size of tumoral nodules was observed in exercised mice. Notably, regular exercise had only minor effects on metabolic and physiologic parameters in *AlbCrePten*$^{flox/flox}$ mice and the beneficial effect of regular exercise on tumor development was independent of any histological improvement in steatosis or NASH. The impairment in activity of the complex mTOR-Raptor provides one mechanism explaining the favorable effect of regular exercise on tumoral growth.

Ablation of the *Pten* gene in mouse hepatocytes results in the spontaneous development of steatohepatitis in animals older than 10 weeks of age, followed by spontaneous tumorigenesis in liver of mice older than 40 weeks. This NAFLD background of hepatic tumorigenesis provided a convenient model for the investigation of the effect of regular exercise on the development of HCC. Regular exercise was started at the age of 7–9 weeks, when liver of *AlbCrePten*$^{flox/flox}$ mice show signs of steatosis, and exercise was continued for 32 weeks, until an age where animals presented hepatic tumors. Treadmill exercise reduced the number and the size of liver nodules in *AlbCrePten*$^{flox/flox}$ mice, indicating that exercise was able to slow down the progression of liver carcinogenesis in our NAFLD model. Several studies have already demonstrated the impact of swimming on tumoral growth [22, 23]. Interestingly, Aguiar e Silva et al. demonstrated that swimming attenuated chemically induced liver carcinogenesis in Wistar rats, although the animals were under a reduced fat diet [23]. Physical activity has been demonstrated to impact spontaneous cancer progression in other organs such as prostate, breast and intestine [24-26]. Our rodent study is, however, the first to present an impact of

regular exercise on spontaneous hepatic tumor progression in an NAFLD environment.

A variety of physiologic processes, including exercise, activates AMPK. The liver is highly sensitive to metabolic demands during muscular work [19, 27] and AMPK activation has been shown to increase following short- and long-term exercise in rat liver [19, 28, 29]. The AMPK-mTORC1 signaling pathway is involved in growth suppression and hepatocarcinogenesis [30], and activation of this signaling pathway induces cell cycle arrest and apoptosis [21]. AMPK acts by direct phosphorylation of the tuberous sclerosis complex 2 (TSC2) tumor suppressor at Thr1227 and Ser345, enhancing its GTP-ase activity towards Rheb (Ras homolog enriched in brain), resulting in inactivation of Rheb and in decreased mTORC1 signaling [31]. In addition, AMPK can also directly phosphorylate the mTORC1 component Raptor on Ser722 and Ser792, inducing 14-3-3 binding to Raptor, resulting in the inhibition of the mTORC1 activity and cell cycle arrest [20]. In our study, the activation of AMPK was increased immediately after exercise in our acute exercise experiment, which was accompanied by increased phosphorylation of Raptor at site Ser792, the targeted site of AMPK, which results in decreased mTORC1 activity. This reduced activity was further confirmed by the decreased phosphorylation of the S6 ribosomal protein. Even if no difference in the phosphorylation of AMPK, raptor and of the S6 ribosomal protein was observed in liver of the long-term exercise animals due to the 72 hours of rest between the last exercise session and the sacrifice (data not shown), cell proliferation, assessed by Ki67, was reduced by regular exercise in both liver and tumoral tissues. These data are consistent with previous studies in different cancer models showing that pharmacologic activation of the AMPK signaling by metformin, AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide) or phenformin, may attenuate cancer cell growth through cell cycle arrest and decreased cell proliferation

[32-34]. In each of these studies, cell cycle arrest was associated with an increased expression of the p21 cell cycle inhibitor. In our animal model, the expression of the *p21* gene (*Cdkn1a*) was increased shortly after a single bout of exercise, as a consequence of AMPK signaling pathway activation (data not shown). It is possible that the repetition of acute activation of AMPK in hepatic tissue is able to decrease over the long term hepatic and tumoral cell proliferation through acute regulation of genes involved in cell growth. We also showed that the expression of *Ddit4* was increased immediately after exercise. DDIT4 (also known as REDD1 (Regulated in development and DNA damage response 1)) is a negative regulator of TORC1 signaling, the expression of which is induced in response to many stresses such as hypoxia, DNA damage, oxidative stress, energy depletion or glucocorticoid treatment. These results suggest that upregulation of AMPK is not the only pathway involved in the decrease in mTORC1 activity during exercise. In addition to its involvement in cell cycle arrest and apoptosis, mTORC1 is also a key complex in the regulation of autophagy. Autophagy includes all processes by which cytoplasmic materials, including organelles, reach lysosomes for degradation [35]. In the liver, autophagy is involved in liver physiology and metabolism [36]. It is also implicated in liver pathology, such as NAFLD, since autophagy possesses a role in the removal of lipid droplets from hepatocytes, or HCC, and is described as a tumor suppressor mechanism in this pathology [35]. Activation of mTORC1, as observed in the hepatocytes of our *AlbCrePten*$^{flox/flox}$ mice, results in inhibition of autophagy, and activation of AMPK may stimulate autophagy by inhibiting mTORC1 activity, thus preventing tumoral growth. Indeed, data have showed reduced autophagy in HCC [35], and the AMPK activator metformin may be associated with reduced HCC risk in patients with diabetes, and slower progression of HCC development [37, 38]. We were unable to demonstrate any induction of autophagy in the liver tissue of animals

353 sacrificed immediately after the end of an exercise session (data not shown). Bayod
354 et al [39] demonstrated similar results in liver of rats after long-term moderate
355 training. Thus the effect of exercise on HCC development observed in our
356 *AlbCrePten*$^{flox/flox}$ mice does not seem to involve autophagy despite its effect on the
357 activation of AMPKα and the inhibition of mTORC1.
358 The release from working muscles of several myokines which exert paracrine or
359 endocrine effects on different organs, may also contribute to the decrease of cell
360 proliferation observed in liver and tumoral tissue. Indeed some of these myokines
361 have been shown to inhibit tumor cell growth [40], suggesting the role of muscle-
362 released factors in cancer protection. Furthermore, exercise-released myokines may
363 mediate direct anti-inflammatory effects on the liver and contribute to tumor
364 protection [41]. Further investigations are needed to confirm the role of myokines in
365 the prevention of tumor growth, for example by studying the effect of serum collected
366 from animals immediately after a single bout of exercise on hepatocarcinoma cell
367 growth.
368 The beneficial effect of regular exercise on tumor development was independent of
369 the improvement of steatosis and NASH lesions, since exercise was unable over the
370 long term to reduce hepatic triglyceride content and to improve liver injury induced by
371 steatosis in *AlbCrePten*$^{flox/flox}$ mice, despite an effect on fatty acid metabolism
372 observed immediately after the end of a training session. These data are also in
373 contradiction with those showed by Kawanishi et al. who demonstrated that exercise
374 attenuated NAS in diet-induced obese C57BL/6 mice [18]. In contrast to this model,
375 where steatosis and liver lesions were induced by feeding healthy animals with a
376 special diet, *AlbCrePten*$^{flox/flox}$ mice show high expression level of genes involved in
377 lipid synthesis and develop spontaneously steatosis and steatohepatitis without any
378 external treatment [16]. The steatosis score and NAS observed in these animals

represented the basal levels and it is possible that the intensity of the exercise sessions used in our study was not sufficient to further reduce steatosis and NAS caused by the genetic deletion of *Pten*. Indeed the effect on lipogenic proteins was weak and observed only immediately after the end of exercise session (Supplementary Fig.5), no difference in enzymes involved in lipogenesis being observed in mice sacrificed 72 hours after the last training session (data not shown). We also studied the effects of exercise on glucose metabolism in *AlbCrePten*$^{flox/flox}$ mice, by performing a glucose tolerance test and measuring blood glucose levels. Several studies demonstrated a beneficial effect of regular exercise on blood glucose level, glucose tolerance and insulin sensitivity in different diet-induced fatty-liver mice models [42-44]. However, we were unable to demonstrate any advantageous effect of regular exercise on glucose metabolism in *AlbCrePten*$^{flox/flox}$ mice, as shown by fasted glucose level or glucose tolerance test, despite a slight improvement in the body weight. *AlbCrePten*$^{flox/flox}$ mice are characterized by insulin hypersensitivity and improved glucose tolerance compared with wild-type animals [16], and this is the probable reason why exercise did not show any impact on glucose metabolism in our study. In another murine model of steatohepatitis characterized by insulin resistance (FXR$^{-/-}$ mice) [45], glucose tolerance was improved by our exercise protocol (data not shown).

Yamaguchi et al. demonstrated that decreased liver injury can be independent of steatosis improvement. These authors suggest even that accumulation of triglycerides may be a protective mechanism to prevent progressive liver damage. They demonstrated that inhibition of triglyceride synthesis not only decreased hepatic steatosis and improved systemic insulin sensitivity, but also increased hepatic free fatty acids content, oxidative stress, hepatocellular apoptosis lobular inflammation and fibrosis [46]. Moreover there is evidence that the quality of lipids plays a more

important role than the quantity in the risk of progressive diseases [47]. Exercise may thus modify the type of lipids accumulated in the liver and makes them less harmful to hepatocytes. Further studies would be needed to study the effect of exercise on the quality of hepatic lipids.

RNA-Seq analysis was performed on liver tissue of mice sacrificed immediately after exercise. This analysis showed that pathways involved in fatty acid metabolism were significantly affected. However no pathways involved in cell proliferation, cell growth and carcinogenesis were found to be altered immediately after exercise. One explanation could be that the intensity used in our animal model was probably not sufficient enough to impact such pathways in our mice characterized by a genetic deletion of a tumor suppressor. It should also be emphasized that the analysis done here were performed on liver tissue collected immediately after the end of training. Exercise can also induce adaptive response during the recovery phase which may affect different molecular mechanisms involved in cell proliferation and cell growth. The effect of repetitive activation of pathways on the long term should not be forgotten. Finally, the analyses were performed on whole liver tissue extracts, which might also explain why no alteration in cell proliferation, cell growth and carcinogenesis pathways could be observed. Further studies investigating carefully specific changes induced by exercise in tumoral tissues versus non-tumoral tissues are needed to further elucidate the molecular effects of exercise in the prevention of HCC development.

The exercise regime used in the present study is of low-to-moderate intensity, as the treadmill training corresponds to 70% of the maximum aerobic capacity [48], accompanied by a slight increase of lactate from 1.5 mM in sedentary mice to 3 mM in exercise mice after 1 hour of exercise (data not shown). In a breast cancer model in rats, low-intensity running showed no effect on carcinogenesis whereas higher

exercise intensity resulted in a decreased incidence of mammary cancer [49]. Similarly, anaerobic physical activity, but not aerobic, was described to reduce the incidence of experimental lung tumors in mice [50]. In our study, an effect on tumoral growth was already observed with a low-to-moderate exercise protocol. This is important from a clinical point of view, since NAFLD patients with high risk to develop HCC are mostly obese or diabetic. It is possible to encourage such patients to increase the amount of exercise; however, it is unlikely they will be able to sustain regular high-intensity exercise.

In conclusion, this study is the first to show a beneficial effect of regular exercise on the development of liver tumors in a NAFLD environment. Lifestyle modifications, which include weight loss and exercise, are the best preventive and curative measures for NAFLD and associated metabolic diseases, such as diabetes and obesity. Therefore, these data should further encourage such patients to undertake all measures for increasing their physical activity.

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FIGURES LEGENDS

Fig. 1. Effect of exercise on the development of liver nodules in *AlbCrePten$^{flox/flox}$* mice. (A) Representative pictures of liver from *AlbCrePten$^{flox/flox}$* mice performing regular exercise or remaining sedentary. A significantly lower number of exercise mice developed nodules (*p<0.05), whereas nodules were observed in all of the sedentary mice. (B) Distribution between the total volume of nodules per liver and the number of mice (*p<0.05).

Fig. 2. Liver histology in *AlbCrePten$^{flox/flox}$* mice performing regular exercise. (A) Magnification (200x) of hematoxylin and eosin (H&E)-stained liver sections in *AlbCrePten$^{flox/flox}$* animals, showing no difference in lipid vacuoles between regular exercise and sedentary mice. Scale = 100 µM. (B) Steatosis score and NAFLD Activity Score (NAS) determined by histologic evaluation according to Kleiner [17].

Fig. 3. Effect of exercise on AMPK-mTOR signaling. Left: Immunoblots for the phosphorylation of AMPKα (A), Raptor (B) and S6 (C) proteins in liver extracts of *AlbCrePten$^{flox/flox}$* mice sacrificed 15 minutes after a single bout of exercise or remaining sedentary. *β*-Actin was used as loading control. Right: The immunoblots were quantified and the ratios p-AMPKα/AMPKα, p-raptor/raptor and p-S6/S6 calculated. The ratios were normalized to sedentary values (*p<0.05). (D) qPCR analysis of *Ddit4* mRNA levels in liver of *AlbCrePten$^{flox/flox}$* mice sacrificed 15 minutes after a single bout of exercise or remaining sedentary. Data were normalized to sedentary values (*p<0.05).

Fig. 4. Effect of exercise on cell proliferation in tumoral nodules sections. Left: Representative 200x magnification images of Ki67 immunostaining in tumor sections

larger than 15 mm^3 (A) or in nodules smaller than 15 mm^3 present in liver sections (B) of *AlbCrePten*$^{flox/flox}$ mice performing regular exercise or remaining sedentary. Scale = 100 µM. Right: Quantification of Ki67-positive cells per mm^2 in tumor larger than 15 mm^3 (A) or in nodules smaller than 15 mm^3 (B) using the Metamorph software (*$p<0.05$; #$p=0.06$).

Fig 5. Effect of exercise on metabolic pathways. Heat maps showing gene expression increased (red) and decreased (blue) in a comparison of liver extract from animals sacrificed immediately after a single bout of exercise versus sedentary mice. (A), Fatty acid, triacylglycerol and ketone body metabolism, (B) Fatty acid β oxidation, (C) PPAR signalling pathway, (D) Regulation of cellular ketone metabolic process, (E) NAFLD and (F) PI3K Akt signalling.

Figure 1

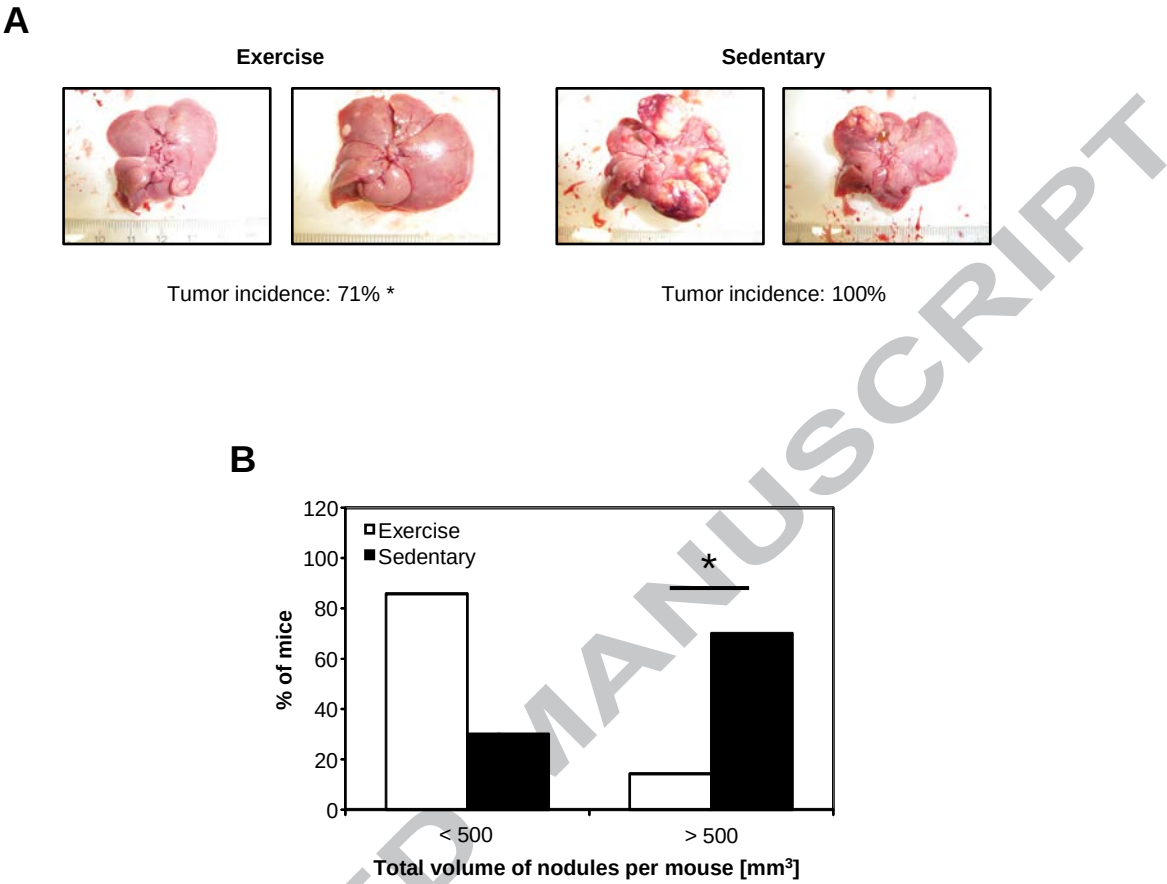

Figure 2

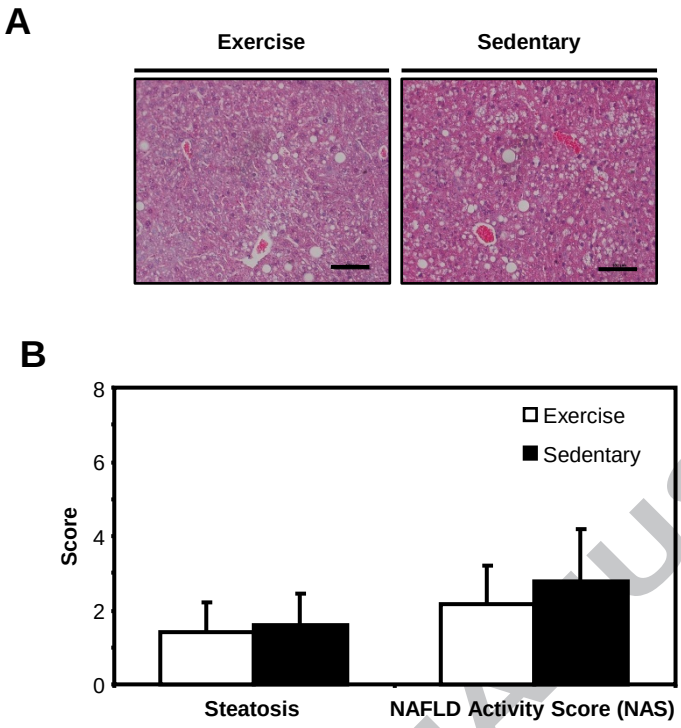

Figure 3

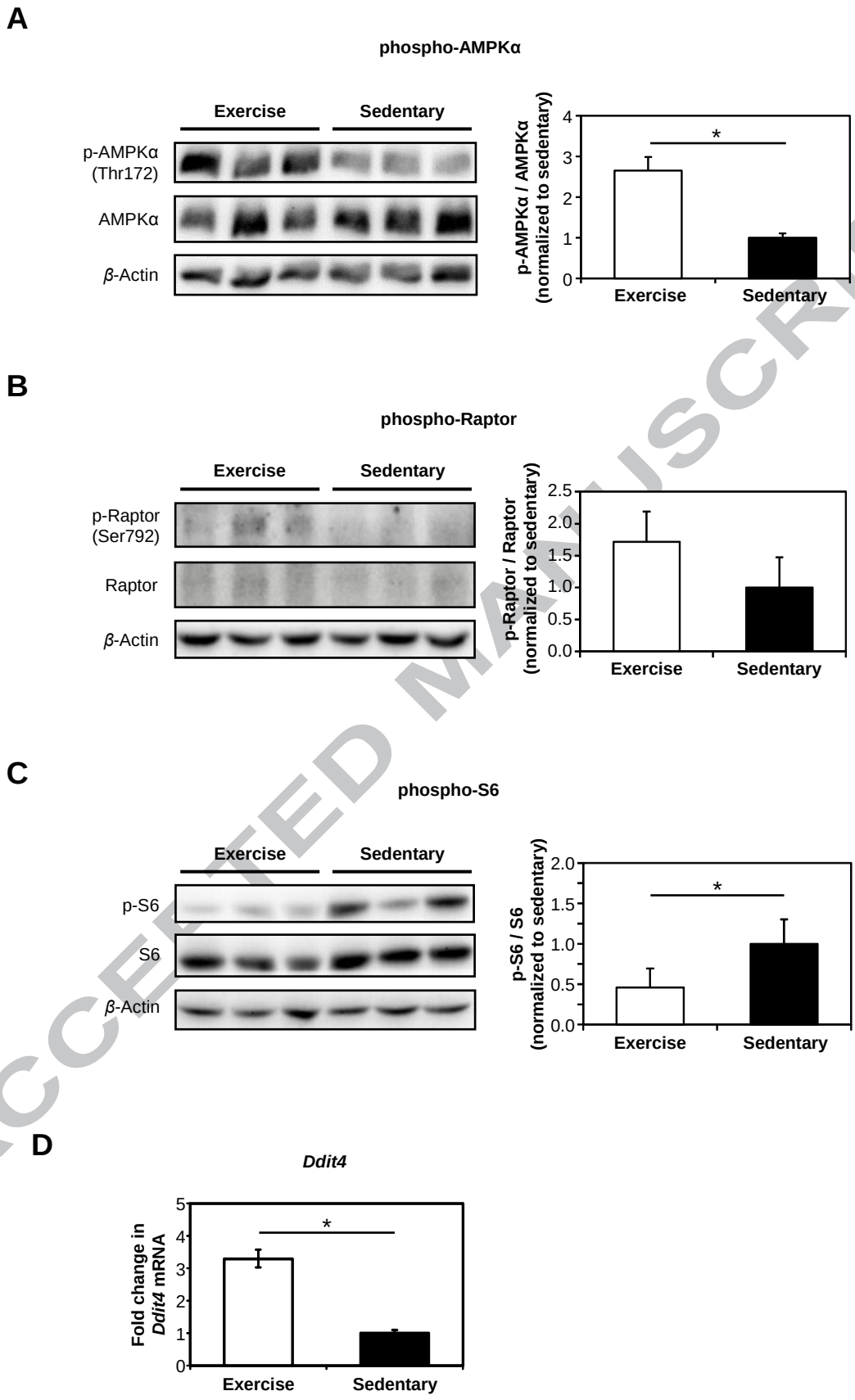

Figure 4

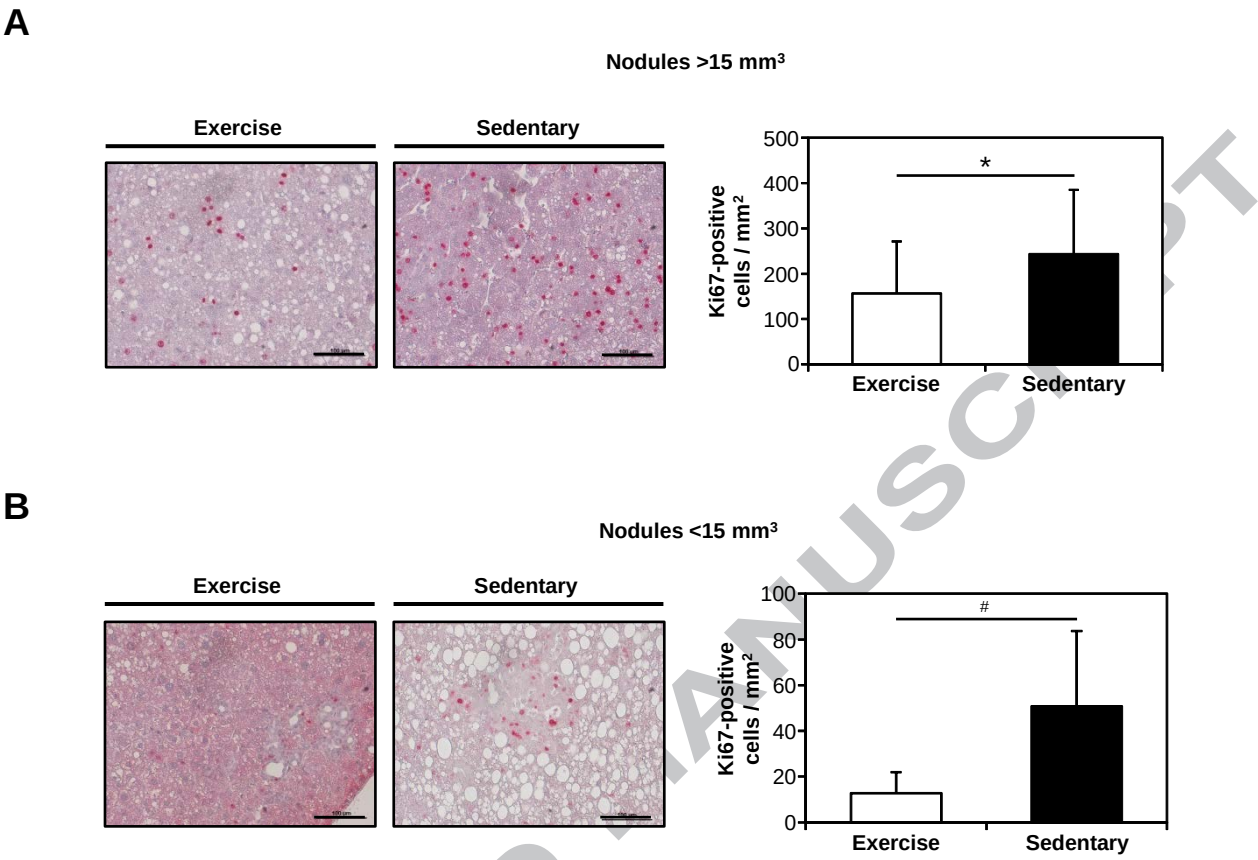

Figure 5