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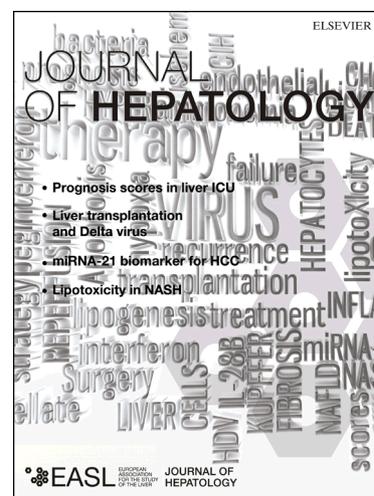
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1 **Regular exercise decreases liver tumors development in hepatocyte-specific**
2 **PTEN-deficient mice independently of steatosis**

3

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26

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34 **List of abbreviations:**

35 HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; NASH,

36 non-alcoholic steatohepatitis; PTEN, phosphatase and tensin homolog deleted from

37 chromosome 10; NAS, NAFLD activity score; qPCR, Real-Time Quantitative

38 Polymerase Chain Reaction; AMPK, AMP-activated protein kinase; mTOR,

39 mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex

40 1; Ddit4, DNA-damage-inducible transcript 4; ACC, Acetyl-CoA carboxylase; FAS,

41 Fatty acid synthase

42

43 **Keywords:**

44 Non-alcoholic fatty liver disease; non-alcoholic steatohepatitis; hepatocellular

45 carcinoma; AMPK; mTOR.

46

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48 The authors who have taken part in this study declared that they do not have any

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51

52

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58

59 **Authors contributions:**

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

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

64 Cedric Simillion: analysis and interpretation of data; statistical analysis

65 Irene Keller: analysis and interpretation of data; statistical analysis

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

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

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

73 **ABSTRACT**

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

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

84 **Results:** After 32 weeks of regular exercise, 71% of exercised mice developed
85 nodules larger than 15 mm³ vs 100% of mice in the sedentary group. The mean
86 number of tumors per liver was reduced by exercise, as well as the total tumoral
87 volume per liver. Exercise did not affect steatosis and had no effect on the Non-
88 alcoholic fatty liver disease (NAFLD) Activity Score (NAS). Exercise decreased tumor
89 cell proliferation. Mechanistically, exercise stimulated the phosphorylation of AMPK
90 and its substrate raptor, which decreased the kinase activity of mTOR.

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

94

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96

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98 hepatocellular carcinoma; AMPK; mTOR.

99 **INTRODUCTION**

100 With more than half a million new cases diagnosed each year in the world and with a
101 similar number of deaths, liver cancer is the fifth most commonly diagnosed cancer in
102 the world and the second most common cause of cancer-related mortality [1].
103 Hepatocellular carcinoma (HCC) represents the major primary malignancy of the liver
104 [2], with an incidence rate that is growing in the Western World [3]. The main
105 reasons for this increase are the epidemic of hepatitis C virus, alcohol abuse, and the
106 surge in obesity [1-3]. Moreover, growing evidence supports the role of non-alcoholic
107 fatty liver disease (NAFLD) and its complication, non-alcoholic steatohepatitis
108 (NASH), as risk factors for HCC [4]. NAFLD, characterized by excessive fat
109 accumulation in the liver, termed steatosis, is the most common liver disease in
110 developed countries. The disease can progress to NASH with the appearance of
111 histologic features of hepatocellular inflammation, ballooning, Mallory-Denk bodies
112 and fibrosis. NAFLD is strongly associated with the prevalence of obesity and type 2
113 diabetes [5], and both diseases are established as major risk factors for the
114 development of HCC [6, 7]. Lifestyle changes, which include weight loss and
115 increasing physical activity, are the best preventive and curative measures against
116 obesity and diabetes, and several studies have demonstrated the beneficial effect of
117 physical activity in the prevention of the progression of NAFLD to NASH [8].
118 Accumulating evidence suggests that physical activity and regular exercise provide
119 other health benefits, including relief of cancer treatment-related symptoms, such as
120 fatigue [9], but also protection against cancer and improved survival in those with
121 cancer. It has recently been demonstrated that physical inactivity is associated with
122 5% to 12% of breast and colon cancers [10], while for breast cancer sufferers,
123 physical activity has been shown to be associated with decreased incidence and
124 reduced risk for recurrence and mortality [11, 12]. Similarly, a reduced risk and

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

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

135 **MATERIAL AND METHODS**

136 **Animals and dietary treatment**

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

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

148

149 **Exercise protocol**

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

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

166

167 Further methods are described in Supplementary Material.

168 **RESULTS**

169 **Effect of exercise on the development of tumoral nodules**

170 The incidence of tumoral nodule development in *AlbCrePten*^{flox/flox} mice was
171 significantly reduced by exercise, with 71% of exercised mice developing liver
172 nodules, compared to 100% of sedentary mice (Fig. 1A). As shown in Fig. 1A, the
173 mean number of nodules larger than 15 mm³ per liver was reduced by exercise
174 (1.8±0.8 vs 2.8±2.3). In addition, measurement of the size of the nodules larger than
175 15 mm³ and calculation of the total volume of nodules per liver showed that the
176 combined volume in liver of exercised mice was less than half of that of mice in the
177 sedentary group (444±551 vs 945±1007). The distribution between the total volume
178 of tumoral nodules per liver and the number of mice (Fig. 1B) confirmed that a
179 significantly higher number of sedentary *AlbCrePten*^{flox/flox} mice developed larger
180 nodules compared with the exercised mice.

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

183

184 **Effects of exercise on metabolic and physiologic parameters**

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

193 There was a significant decrease in the body weight of mice after 32 weeks of regular
194 exercise ($p=0.03$; Supplementary Fig. 2B). The weight gain between the first and the
195 last week of exercise was also significantly reduced ($p=0.005$; Supplementary Fig.
196 2C). There was a trend ($p=0.06$) toward a reduction in the epididymal fat mass in
197 regularly exercised mice (Supplementary Fig. 2D). In contrast, the liver weight and
198 the liver weight per body weight ratio were not significantly affected after 32 weeks of
199 exercise (Supplementary Fig. 2E-F).

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

204

205 **Effect of exercise on liver steatosis and hepatic injury**

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

216

217 **Effect of exercise on AMPK-mTOR signaling**

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

239 The mTORC1 complex, formed among others of mTOR and Raptor, is involved in
240 cell growth and cell proliferation[21]. Therefore, cell proliferation was assessed by
241 Ki67 immunohistostaining in nodules larger and smaller than 15 mm³ observed in
242 liver of *AlbCrePten^{flox/flox}* mice. Cell proliferation in nodules larger than 15 mm³ was
243 significantly decreased by exercise (p=0.036) and showed a trend towards reduction

244 in nodules smaller than 15 mm³ present in liver tissue ($p=0.06$; Fig. 4). The number
245 of Ki67-positive cells in liver tissue was also significantly decreased by regular
246 exercise compared with no exercise (data not shown). Taken together, these results
247 suggested that regular exercise led to a decrease of hepatocellular cell proliferation
248 in *AlbCrePten*^{flox/flox} mice. This effect could partly be explained by the decrease of
249 mTORC1 activity induced by the repetitive impact of exercise on AMPK activity and
250 on *Ddit4* gene expression, leading to a decrease of tumor growth.

251

252 **Effect of exercise on metabolism signaling**

253 It is well known that exercise induces many beneficial metabolic effects. To
254 understand the effect of exercise on metabolic pathways in *AlbCrePten*^{flox/flox} mice,
255 RNA-Seq analysis was performed on liver tissue of animals sacrificed immediately
256 after a single bout of exercise or after sedentariness. The DESeq2 software was
257 used to calculate differential expression between genes in the exercised group and
258 the sedentary group. To detect which pathways are specifically affected by exercise,
259 a gene set enrichment analysis (GSEA) was performed on the output of DESeq2
260 using the newly developed SetRank method (Supplementary Table 4). By applying
261 more stringent parameters, 6 pathways were found to be significantly altered.
262 Interestingly, these pathways were mostly involved in fatty acid metabolism (Fig. 5).
263 AMPK activation is known to phosphorylate and inactivate a number of metabolic
264 enzymes involved in lipid metabolism, especially ACC, a key enzyme in fatty acid
265 synthesis whose phosphorylation by AMPK leads to its enzymatic inactivation [19].
266 The phosphorylation of ACC at the AMPK target site Serine 79 was increased in the
267 liver tissue *AlbCrePten*^{flox/flox} mice immediately after the end of the exercise session
268 (Supplementary Fig. 4), confirming among others the effect of exercise on AMPK
269 activation. The inhibition of the enzymatic activity of ACC was accompanied by a

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

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

275 **DISCUSSION**

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

285 Ablation of the *Pten* gene in mouse hepatocytes results in the spontaneous
286 development of steatohepatitis in animals older than 10 weeks of age, followed by
287 spontaneous tumorigenesis in liver of mice older than 40 weeks. This NAFLD
288 background of hepatic tumorigenesis provided a convenient model for the
289 investigation of the effect of regular exercise on the development of HCC. Regular
290 exercise was started at the age of 7–9 weeks, when liver of *AlbCrePten^{flox/flox}* mice
291 show signs of steatosis, and exercise was continued for 32 weeks, until an age
292 where animals presented hepatic tumors. Treadmill exercise reduced the number
293 and the size of liver nodules in *AlbCrePten^{flox/flox}* mice, indicating that exercise was
294 able to slow down the progression of liver carcinogenesis in our NAFLD model.

295 Several studies have already demonstrated the impact of swimming on tumoral
296 growth [22, 23]. Interestingly, Aguiar e Silva et al. demonstrated that swimming
297 attenuated chemically induced liver carcinogenesis in Wistar rats, although the
298 animals were under a reduced fat diet [23]. Physical activity has been demonstrated
299 to impact spontaneous cancer progression in other organs such as prostate, breast
300 and intestine [24-26]. Our rodent study is, however, the first to present an impact of

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

303 A variety of physiologic processes, including exercise, activates AMPK. The liver is
304 highly sensitive to metabolic demands during muscular work [19, 27] and AMPK
305 activation has been shown to increase following short- and long-term exercise in rat
306 liver [19, 28, 29]. The AMPK-mTORC1 signaling pathway is involved in growth
307 suppression and hepatocarcinogenesis [30], and activation of this signaling pathway
308 induces cell cycle arrest and apoptosis [21]. AMPK acts by direct phosphorylation of
309 the tuberous sclerosis complex 2 (TSC2) tumor suppressor at Thr1227 and Ser345,
310 enhancing its GTP-ase activity towards Rheb (Ras homolog enriched in brain),
311 resulting in inactivation of Rheb and in decreased mTORC1 signaling [31]. In
312 addition, AMPK can also directly phosphorylate the mTORC1 component Raptor on
313 Ser722 and Ser792, inducing 14-3-3 binding to Raptor, resulting in the inhibition of
314 the mTORC1 activity and cell cycle arrest [20]. In our study, the activation of AMPK
315 was increased immediately after exercise in our acute exercise experiment, which
316 was accompanied by increased phosphorylation of Raptor at site Ser792, the
317 targeted site of AMPK, which results in decreased mTORC1 activity. This reduced
318 activity was further confirmed by the decreased phosphorylation of the S6 ribosomal
319 protein. Even if no difference in the phosphorylation of AMPK, raptor and of the S6
320 ribosomal protein was observed in liver of the long-term exercise animals due to the
321 72 hours of rest between the last exercise session and the sacrifice (data not shown),
322 cell proliferation, assessed by Ki67, was reduced by regular exercise in both liver and
323 tumoral tissues. These data are consistent with previous studies in different cancer
324 models showing that pharmacologic activation of the AMPK signaling by metformin,
325 AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide) or phenformin, may
326 attenuate cancer cell growth through cell cycle arrest and decreased cell proliferation

327 [32-34]. In each of these studies, cell cycle arrest was associated with an increased
328 expression of the p21 cell cycle inhibitor. In our animal model, the expression of the
329 *p21* gene (*Cdkn1a*) was increased shortly after a single bout of exercise, as a
330 consequence of AMPK signaling pathway activation (data not shown). It is possible
331 that the repetition of acute activation of AMPK in hepatic tissue is able to decrease
332 over the long term hepatic and tumoral cell proliferation through acute regulation of
333 genes involved in cell growth. We also showed that the expression of *Ddit4* was
334 increased immediately after exercise. DDIT4 (also known as REDD1 (Regulated in
335 development and DNA damage response 1)) is a negative regulator of TORC1
336 signaling, the expression of which is induced in response to many stresses such as
337 hypoxia, DNA damage, oxidative stress, energy depletion or glucocorticoid treatment.
338 These results suggest that upregulation of AMPK is not the only pathway involved in
339 the decrease in mTORC1 activity during exercise. In addition to its involvement in cell
340 cycle arrest and apoptosis, mTORC1 is also a key complex in the regulation of
341 autophagy. Autophagy includes all processes by which cytoplasmic materials,
342 including organelles, reach lysosomes for degradation [35]. In the liver, autophagy is
343 involved in liver physiology and metabolism [36]. It is also implicated in liver
344 pathology, such as NAFLD, since autophagy possesses a role in the removal of lipid
345 droplets from hepatocytes, or HCC, and is described as a tumor suppressor
346 mechanism in this pathology [35]. Activation of mTORC1, as observed in the
347 hepatocytes of our *AlbCrePten^{flox/flox}* mice, results in inhibition of autophagy, and
348 activation of AMPK may stimulate autophagy by inhibiting mTORC1 activity, thus
349 preventing tumoral growth. Indeed, data have showed reduced autophagy in HCC
350 [35], and the AMPK activator metformin may be associated with reduced HCC risk in
351 patients with diabetes, and slower progression of HCC development [37, 38]. We
352 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 AMPK α 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

379 represented the basal levels and it is possible that the intensity of the exercise
380 sessions used in our study was not sufficient to further reduce steatosis and NAS
381 caused by the genetic deletion of *Pten*. Indeed the effect on lipogenic proteins was
382 weak and observed only immediately after the end of exercise session
383 (Supplementary Fig.5), no difference in enzymes involved in lipogenesis being
384 observed in mice sacrificed 72 hours after the last training session (data not shown).
385 We also studied the effects of exercise on glucose metabolism in *AlbCrePten^{flox/flox}*
386 mice, by performing a glucose tolerance test and measuring blood glucose levels.
387 Several studies demonstrated a beneficial effect of regular exercise on blood glucose
388 level, glucose tolerance and insulin sensitivity in different diet-induced fatty-liver mice
389 models [42-44]. However, we were unable to demonstrate any advantageous effect
390 of regular exercise on glucose metabolism in *AlbCrePten^{flox/flox}* mice, as shown by
391 fasted glucose level or glucose tolerance test, despite a slight improvement in the
392 body weight. *AlbCrePten^{flox/flox}* mice are characterized by insulin hypersensitivity and
393 improved glucose tolerance compared with wild-type animals [16], and this is the
394 probable reason why exercise did not show any impact on glucose metabolism in our
395 study. In another murine model of steatohepatitis characterized by insulin resistance
396 (*FXR^{-/-}* mice) [45], glucose tolerance was improved by our exercise protocol (data not
397 shown).
398 Yamaguchi et al. demonstrated that decreased liver injury can be independent of
399 steatosis improvement. These authors suggest even that accumulation of
400 triglycerides may be a protective mechanism to prevent progressive liver damage.
401 They demonstrated that inhibition of triglyceride synthesis not only decreased hepatic
402 steatosis and improved systemic insulin sensitivity, but also increased hepatic free
403 fatty acids content, oxidative stress, hepatocellular apoptosis lobular inflammation
404 and fibrosis [46]. Moreover there is evidence that the quality of lipids plays a more

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

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

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

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

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

445

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599

600

601 **FIGURES LEGENDS**

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

608

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

614

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

624

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

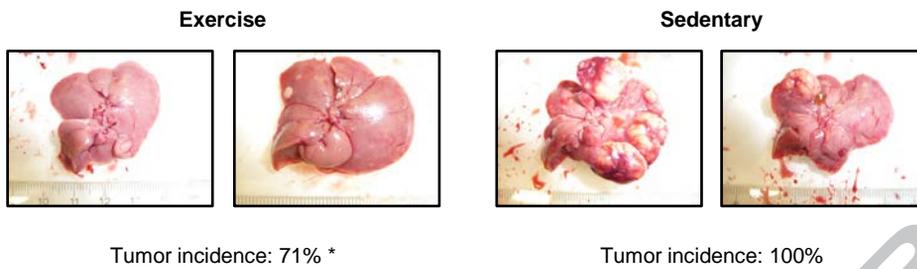
627 larger than 15 mm³ (A) or in nodules smaller than 15 mm³ present in liver sections (B)
628 of *AlbCrePten^{flox/flox}* mice performing regular exercise or remaining sedentary. Scale =
629 100 μM. Right: Quantification of Ki67-positive cells per mm² in tumor larger than 15
630 mm³ (A) or in nodules smaller than 15 mm³ (B) using the Metamorph software
631 (*p<0.05; #p=0.06).

632

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

Figure 1

A



B

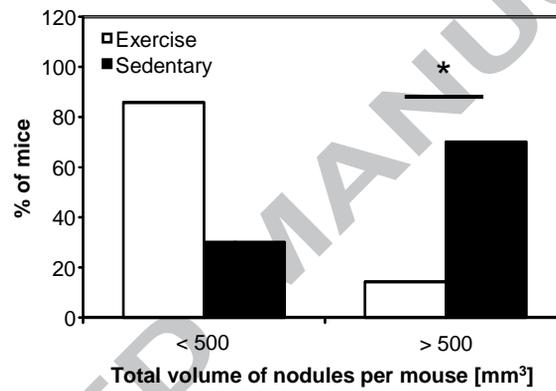


Figure 2

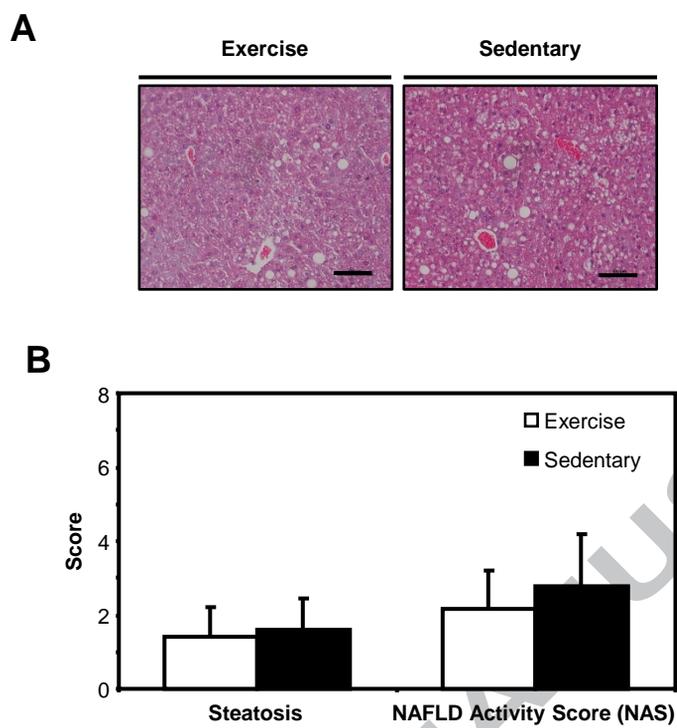
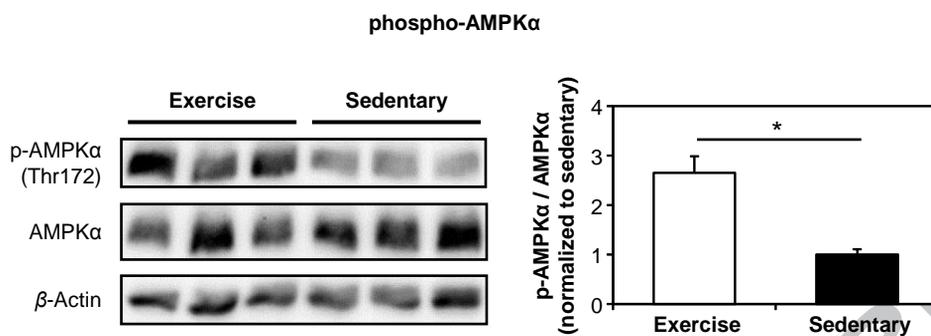
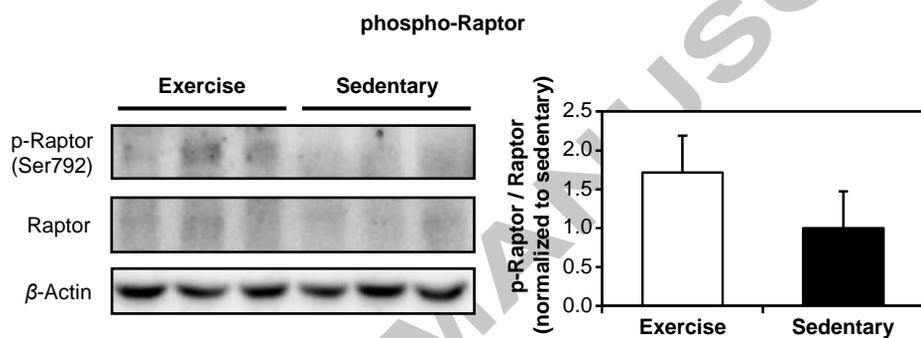


Figure 3

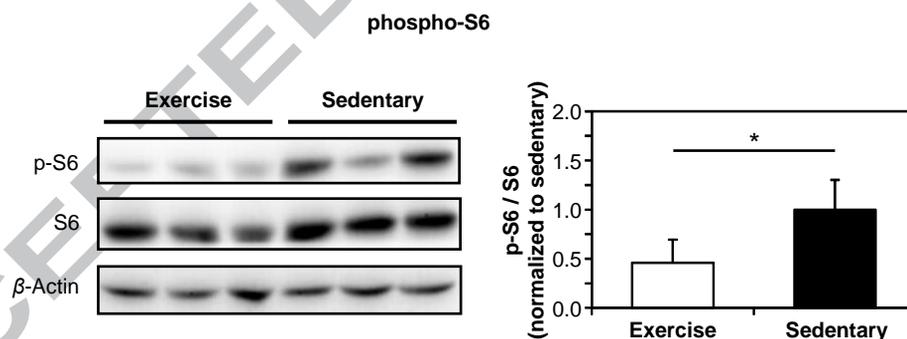
A



B



C



D

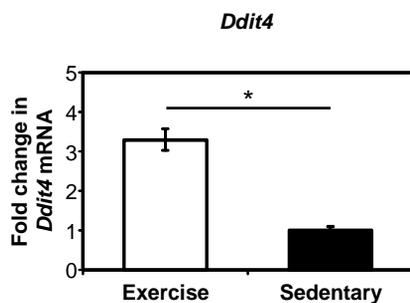


Figure 4

