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Early intervention exercise training does not delay prostate cancer progression in *Pten*$^{-/-}$ mice

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Abstract

Background: There is convincing evidence that men with advanced prostate cancer experience improved quality of life as a result of exercise therapy, although there is limited pre-clinical, and no clinical, data to directly support the notion that exercise training improves prostate cancer prognosis or outcome. The aim of this study was to investigate the effect of regular exercise training on the early stages of prostate cancer progression, as well as assessing whether alterations to prostate cancer metabolism are induced by exercise.

Methods: Mice with prostate-specific deletion of *Pten* (*Pten*$^{-/-}$) remained sedentary or underwent six-weeks of endurance exercise training or high-intensity exercise training involving treadmill running. At the conclusion of the training period, the prostate lobes were excised. A portion of fresh tissue was used to assess glucose, glutamine and fatty acid metabolism by radiometric techniques and a second portion was fixed for histopathology.

Results: Despite the implementation of an effective exercise regime, as confirmed by improvements in running capacity, neither prostate mass, cell proliferation or the incidence of high-grade prostate intraepithelial hyperplasia or non-invasive carcinoma *in situ* were significantly different between groups. Similarly, neither glucose uptake, oxidation and *de novo* lipogenesis, glutamine oxidation, or fatty acid uptake, oxidation and storage into various lipids were significantly different in prostate tissue obtained from untrained and exercise trained mice.

Conclusions: These results show that six weeks of moderate or high-intensity exercise training does not alter substrate metabolism in the prostate or slow the progression of *Pten*-null prostate cancer. These results question whether exercise is a useful therapy to prevent or delay prostate cancer progression.

Introduction

Prostate cancer is the most common cancer in men and the second leading cause of cancer-related mortality [1]. It is a slow growing malignancy, which provides the opportunity for intervention to delay tumor progression and increase life expectancy. Current frontline treatments for newly diagnosed prostate cancer include radical prostatectomy or radiotherapy, while patients with advanced disease typically undergo androgen deprivation therapies (ADT). Each of the treatments inflict significant morbidity, necessitating the need for alternative approaches to treat prostate cancer [2].

A growing body of evidence indicates that lifestyle interventions, such as reduction of dietary fat or total calorie content, and increased physical activity, are linked to reduced incidence and progression of prostate cancer [3-5]. Almost all prospective and case-controlled studies examining the link between exercise training and prostate cancer have

examined the impact of low-intensity and relatively low volume physical activity, and show decreased risk of developing prostate cancer, and lower risk of prostate cancer-related mortality [6-8]. The largest dataset comes from the Health Professionals Follow-up Study, which showed that vigorous activity is associated with a lower risk of advanced, high Gleason grade group, or fatal prostate cancer in men over 65 years of age [9]. Together, these observational studies indicate that physical activity is associated with lower overall prostate cancer mortality, and that vigorous activity may be the most beneficial. There are no clinical trials investigating the effects of exercise on prostate cancer outcomes in men.

In the absence of compelling clinical trial data examining exercise effects on prostate cancer outcomes, direct investigative studies are limited to mice. Prostate cancer progression was delayed by voluntary wheel running activity in a dose dependent manner in C3(1)Tag mice that are predisposed to prostate cancer induced by SV40 oncogenes [10]. However, subsequent studies showed that voluntary running wheel access did not alter primary tumor growth in orthotopically injected transgenic adenocarcinoma of mouse prostate C-1 cells [11] or xenografted LNCaP cells [12]. Alternative approaches have shown a beneficial effect of human post-exercise serum on growth and proliferation of LNCaP and PC3 prostate cancer cell lines [13-16], although the factors mediating such anti-tumorigenic effects are unknown and the physiological relevance of this model unclear. To date, there has been no assessment of vigorous or high-intensity exercise training (HIIT) on prostate cancer progression *in vivo*.

The factors mediating the apparent improvement in prostate cancer prognosis with exercise training remain unresolved, and in most instances, the proposed mechanisms are speculative. Some possibilities include reduced circulating insulin, insulin-like growth

factor 1 and proinflammatory cytokines, reductions in tumor vascularization, androgen receptor adaptations, reduced cholesterol and unknown 'exercise circulating factors' contained in exosomes [17,18]. Recent work has shown that changes in substrate metabolism occur in prostate cancer, with increased reliance on fatty acid uptake and oxidation to fuel cellular ATP production [19] and greater *de novo* lipogenesis for membrane production to facilitate growth [20]. Exercise training is known to modulate substrate metabolism [21], which may explain the purported anti-tumorigenic effects of exercise.

The primary aim of these studies was to determine whether regular moderate-intensity endurance exercise or HIIT slowed the early stages of prostate cancer progression. The secondary aim was to determine whether exercise training altered substrate metabolism in the prostate. We hypothesized that both exercise training modalities would slow the progression of prostate cancer.

Materials and Methods

Animal Studies

All procedures were approved by the Monash Animal Research Platform (MARP) animal ethics committee (MARP/2017/033). Mice were housed at 21°C on a 12:12 h light-dark cycle and were fed a regular chow diet (Specialty Feeds Irradiated Rat and Mouse Pellets; 19.6% energy from protein, 4.6% fat, 4.8% crude fibre, 14.3 MJ/kg energy). Mice expressing probasin cre recombinase (PB-Cre) were crossed with *Pten* floxed mice ($Pten^{flox/flox}$) to generate prostate specific *Pten* knockout mice (referred to herein as $Pten^{-/-}$). All were on a FVBN background. Body mass was assessed weekly.

Exercise Training

The experimental design is shown in Figure 1A. Mice aged five weeks were acclimatized to the rodent treadmill (Colombus Instruments, Ohio, USA) over a period of three days, which involved standing on the treadmill and walking. Mice underwent two tests of exercise capacity. The maximal velocity test involved treadmill running commencing at a velocity of 10 m/min which was increased by 2 m/min every 2 min until mice reached volitional exhaustion. Two days later, mice underwent an endurance capacity test, in which mice ran on the treadmill at a constant speed of 16 m/min until volitional exhaustion. Mice were allocated to one of three experiment groups. Control mice remained sedentary but were exposed to the stationary treadmill for the equivalent time as endurance exercise trained mice. Endurance exercise training constituted a moderate intensity exercise program with progressive overload. Mice commenced training at 12 m/min for 30 min (5% grade), 5 days a week. The velocity and total duration increased weekly such that mice were running at 19 m/min for 70 min during their final week of training. HIIT training involved running at an exercise intensity of 18 m/min (5% grade) for 6 x 2 min bouts, interspersed with 2 min recovery periods, for 5 days / week, with exercise intensity increased by 1 m/min per week, and the number of exercise bouts increased by 1 session per week. Hence, at the conclusion of the 6-week training protocol, mice were running at a velocity of 23 m/min and performing 11 bouts per day, with a maximum total exercise time of 22 mins per session, similar to the protocol described previously [22]. Mice repeated the exercise capacity tests 48 hr after the last exercise bout, as described above.

Tissue Collection

Mice refrained from exercise training for 48 hr prior to tissue collection to wash out any acute exercise effects on metabolism. Mice were fasted from 0700-1100 hr on the day of tissue dissection, then anaesthetized by isoflurane inhalation. Blood was collected by cardiac puncture, added to tubes containing EDTA then spun at 8,000 x *g* for 5 min. The plasma was stored at -80^{o}C for later analysis. Mice were then killed by cervical dislocation. The prostate lobes were carefully isolated, dissected and weighed. The anterior prostate was kept for metabolic analyses (see below), while the lateral prostate was fixed in 10% buffered formalin. The seminal vesicles, liver, and epididymal white adipose tissue dissected and weighed.

Metabolism

A base buffer was prepared for all metabolism experiments and consisted of low glucose (5 mM) Dulbecco's Modified Eagle Medium (DMEM, Gibco, Life Technologies, California, USA), 2 mM glutamine, 1% bovine serum albumin (BSA, Bovogen Biologicals, Melbourne, Australia) and 500 μM oleate (C18:1 fatty acid). The buffer solution was gased with 95% O_2 for 20 min then divided into equal volumes for measurement of fatty acid, glucose and glutamine metabolism.

Fatty acid metabolism. To assess fatty acid metabolism, 1-[^{14}C]oleate (Perkin Elmer, Boston, USA) was added at a concentration of 1 μCi/mL. A piece of anterior prostate (~15 mg) was added to 1 mL of buffer and incubated in a shaking water bath at 35^{o}C for 2 h. At the conclusion of the experiment, prostate tissue was removed from the buffer, washed three times in ice-cold Dulbecco's phosphate-buffered saline, blotted dry and snap frozen in liquid N_2. The tissue was homogenized in 900 μL chloroform: methanol

(2:1 v:v) and 600 μL 0.9% NaOH was subsequently added to separate the organic and aqueous phases, which was facilitated by centrifugation (4°C, 2,000 x *g*, 10 min). The aqueous phase containing the 'incomplete' products of fatty acid oxidation were collected and radioactivity was measured by scintillation counting (Tri Carb 2810TR liquid scintillation analyzer, Perkin Elmer, Boston, USA). The incubation medium was acidified with 1 mL $HClO_4$ and the $^{14}CO_2$ was collected in 300 μL NaOH, which represents 'complete' fatty acid oxidation. This was collected and measured by scintillation counting. Total fatty acid oxidation was calculated by adding 'incomplete' and 'complete' fatty acid oxidation. The organic phase from the tissue homogenization was collected and dried under nitrogen at 40°C. The dried lipids were reconstituted in 1:1 $CHCl_3$ containing triglyceride, diglyceride, phospholipid, cholesterol and ceramide, spotted onto Silica Gel 60 plates then separated by a 3-step thin layer chromatography (TLC) method. The plates were run first in a solution of 65:25:4 chloroform:methanol:water until the solution reached 50% of plate coverage. After being dried for 10 min, the lipids were further separated in 75:35:1 hexane:diethyl ether:acetic acid and resolved to 90%. This step was repeated. The plate was dried, sprayed with dichlorofluorescein dye and the lipid bands were visualized under UV light. Lipids containing the incorporated ^{14}C-oleate were scraped into tubes containing Ultima Gold scintillation fluid and radioactivity assessed. Total fatty acid uptake was calculated by adding fatty acid oxidation to fatty acid storage.

Glucose and Glutamine Metabolism. Preparation of incubation media for measuring glucose and glutamine oxidation was performed identically to fatty acid oxidation, with the exception that D-[^{14}C]-glucose (Perkin Elmer, Boston, USA) or L-[^{14}C]-glutamine (Perkin Elmer, Boston, USA) was added in place of 1-[^{14}C]-oleate. $^{14}CO_2$ was collected for assessment of substrate oxidation. *De novo* lipogenesis was determined by assessing

the incorporation of ^{14}C-glucose or glutamine into free fatty acids, phospholipids and triglycerides using the same TLC method described for fatty acid storage. Glucose uptake was measured over 20 min in isolated prostate tissue using 10 μM 2-deoxyglucose and 1 μCi/mL 2-[1-^{14}C]-deoxyglucose (Perkin Elmer, Boston, USA) [23].

Plasma measurements

Plasma triglycerides (GET KIT; Sigma-Aldrich, Missouri, USA), plasma FFA (NEFA-C ELISA; Wako Life Sciences, California, USA) and plasma insulin (Ultra-Sensitive Mouse Insulin ELISA Kit; Crystal Chem, Illinois, USA) were determined using commercially available assays. Individual samples were assessed in triplicate.

Prostate pathology

Prostate tissues were fixed in 10% buffered formalin for 48 hr, processed and embedded in paraffin. Tissues were cut into 5 μm sections using a microtome. All sections were collected and every 20th section was stained with hematoxylin and eosin (H&E) (Dako, Denmark) in order to determine cancer pathology. Sections were scored as a percentage of tissue with malignant lesions in a blinded manner. Scoring involved evaluating the percentages of tissues that contained normal pathology, low-grade prostatic intraepithelial neoplasia (LG-PIN), high-grade PIN (HG-PIN) or carcinoma *in situ* (CIS) represented as the proportion of the whole tissue. At the post-training time point, when mice were 12-weeks-of-age, regions of carcinoma consisted of non-invasive lesions, where malignant cells were confined to glandular ducts. These lesions were denoted as non-invasive CIS (NI-CIS) (Fig. S1). No metastatic lesions were identified on control or exercise trained mice. The assessment of pathology in *Pten-/-* mice was performed according to the classifications described by Wang and colleagues [24].

To quantitate proliferation, immunohistochemistry was performed on mouse lateral prostate tissues using Ki-67. Staining was performed using the Leica BOND-MAX automated system (Leica Microsystems, Victoria, Australia). Ki-67 primary antibody was incubated for 1 hr at 0.2 μg/mL (Leica Biosystems, MM1, Mouse). Antibody stains for Ki-67 were carried out on three tissue sections per lateral prostate lobe. Slides were converted to digital images using the Aperio ScanScope AT Turbo (Leica Microsystems, Victoria, Australia). Aperio ImageScope analysis software (Version 12.2) was utilized to determine positive cells.

Statistics

Statistical significance was set at $P < 0.05$. All data was analyzed and statistically examined using GraphPad Prism 7 (version 7.0b). Data were first assessed for normal distribution by D'Agostino-Pearson omnibus normality test and data were assessed by two-tailed unpaired t-tests or one-way or two-way analysis of variance with Bonferroni post hoc analysis where appropriate. Non-parametric data were assessed by Dunn's multiple comparisons test. Data is represented as mean ± SEM.

Results

Efficacy of exercise training program

Body mass increased over the duration of the experiment in all groups (Fig. 1B) and there was no significant difference in body mass between groups (Fig. 1B). Consistent with this notion, epididymal fat pad mass was not different between groups at the completion of the study (Table 1). Endurance exercise capacity (Fig. 1C) and maximum running velocity (Fig. 1D) were not significantly different over the course of the experiment in control mice. By contrast, both endurance and HIIT training significantly

improved exercise capacity in mice (Fig. 1C, D), demonstrating the efficacy of both exercise training programs. Plasma NEFA, triglyceride and insulin levels were not significantly different between groups (Table 1).

Effect of exercise training on prostate cancer progression in Pten$^{-/-}$ mice.

Total prostate mass was not different between untrained and exercise trained *Pten*$^{-/-}$ mice (Fig. 2A). Examination of individual prostate lobes showed no effect of exercise training on AP, DP or VP masses, whereas HIIT exercise training significantly reduced lateral prostate mass compared with endurance trained and untrained mice (Fig. 2B). At 12 weeks of age, lateral prostate lobes of *Pten*$^{-/-}$ mice showed predominant regions of HG-PIN (~70%) and non-invasive CIS (~30%) pathology (Fig. 2C), consistent with previously reported *Pten*$^{-/-}$ models on the FVBN background [24]. There was no significant difference in pathology composition in endurance or HIIT exercise groups compared to untrained mice (Fig. 2C). Interestingly, there were foci of low-grade PIN in lateral prostates following endurance exercise that were not observed in control prostates, but this was not a significant change (P = 0.083). Proliferation was assessed in lateral prostate lobes using nuclear Ki-67 localization. The proliferation index was not significantly different between endurance or HIIT exercise and control (Fig. 2D). Collectively, these data indicate that exercise training has no significant effect on prostate cancer progression in *Pten*$^{-/-}$ mice.

Effect of exercise training on metabolism in Pten$^{-/-}$ mouse prostates.

Glucose is the dominant substrate in most cancers [25]. Accordingly, we assessed glucose metabolism in isolated prostate tissue obtained from untrained and exercise trained *Pten*$^{-/-}$ mice. Glucose uptake and oxidation were not different between groups (Fig. 3A, B). *De*

novo lipogenesis is postulated to be important for prostate cancer progression [20]. Neither endurance or HIIT exercise training significantly altered *de novo* lipogenesis in prostate tissue when compared with untrained mice (Fig. 3C).

We used similar approaches to assess glutamine metabolism in isolated prostate tissue. Glutamine oxidation and *de novo* lipogenesis were highly variable between mouse donors, irrespective of training status, and there was no significant difference in glutamine metabolism between groups (Fig. 3D, E).

Recent work indicates increased reliance for fatty acids as a metabolic substrate in prostate cancer [19]. Using ^{14}C-oleate, we show that rates of fatty acid uptake are similar to rates of glucose uptake when assessed on a molar basis (Fig. 4A). Neither fatty acid uptake, fatty acid oxidation, nor incorporation of fatty acids into cholesterol, ceramide, phospholipid, diacylglycerol or triacylglycerol were significantly different between untrained, endurance trained or HIIT trained mice (Fig. 4A-C). It is notable that fatty acid uptake and oxidation were reduced on average by 43% and 44% in the endurance and HIIT groups respectively, but that several outliers in each were responsible for driving this relationship. Altogether, these data indicate that regular exercise training does not significantly alter substrate utilization in murine prostate cancer.

We note that due to limitations in mouse prostate tissue mass, metabolism was assessed in the AP, whereas the LP was used for histopathology. To ascertain whether there were differences in fatty acid metabolism between prostate lobes, we performed additional analysis in four *Pten*$^{-/-}$ mice aged 3 months old. There were no differences between prostate lobes for fatty acid uptake (AP: 1.65 ± 0.19 *vs.* LP: 1.75 ± 0.21 pmol/min/mg, P=0.79), fatty acid oxidation (AP: 0.48 ± 0.07 *vs.* LP: 0.40 ± 0.10 pmol/min/mg, P=0.59) or fatty acid storage into lipids (AP: 1.17 ± 0.15 *vs.* LP: 1.36 ± 0.23 pmol/min/mg,

P=0.58). These data indicate that fatty acid metabolism is similar in anterior and lateral prostate of *Pten*$^{-/-}$ mice.

Discussion

While exercise is proven to improve quality of life for men with advanced prostate cancer who experience secondary effects on muscle, bone and cognitive function as a result of ADT, there is limited epidemiological evidence to indicate that early intervention exercise also improves cancer-specific outcomes for men with prostate cancer. Indeed, the evidence base supporting this is largely confined to retrospective analyses and no clinical trials have formally tested this hypothesis, particularly in men with newly diagnosed or organ-confined disease. Herein, we have designed a randomized pre-clinical trial testing the efficacy of moderate-intensity endurance exercise and high-intensity exercise training on the early stages of prostate cancer progression in *Pten*$^{-/-}$ mice. The results of this study show that exercise, whether moderate- or high-intensity, confers no measurable anti-tumor activity in *Pten*$^{-/-}$ mice.

The results from this mouse study are applicable to patients with localized prostate cancer characterized by *Pten* deletion. Approximately 80% of men have localized disease at diagnosis [26] and ~20% have loss of *Pten* expression [27], indicating broad applicability of this model. We did not observe any significant change in the primary outcome measures of prostate size, tumor pathology or proliferation following 6 weeks of endurance or HIIT exercise training. It is possible that exercise interventions for longer time points may have reduced the tumor burden, especially given there were trends to reduced lateral prostate lobe weight. However, there is documented evidence that other therapeutic interventions can reduce tumor burden in *Pten*$^{-/-}$ mice within this time frame. For example, dual inhibition of RNA Pol I transcription and PIM kinase (CX-6258 plus

CX-5461), reduced cancer progression after just 4 weeks of treatment in *Pten*$^{-/-}$ mice of the equivalent age [28]. Similarly, a diet rich in omega-3 polyunsaturated fatty acids reduced tumor growth, slowed histopathological progression, and increased survival in 8-week-old *Pten*$^{-/-}$ mice [29].

The exercise effects on tumor progression in mice is equivocal. It was previously reported that increasing physical activity by voluntary wheel running delayed prostate cancer progression in C3(1)Tag mice [10], but this effect was not observed in other studies where voluntary wheel running was employed in xenograft or orthotopic prostate cancer cell line models [11,12]. Consistent with these latter studies, we showed that a structured exercise program of treadmill running failed to reduce tumor progression *Pten*$^{-/-}$ mice. The lack of a consistent anti-tumorigenic effect of exercise extends to breast cancer, where most studies report reduced tumorigenesis with exercise training in transgenic mice [30], although a study in *p53*-deficient mice showed that exercise actually accelerated tumorigenesis [31]. Similarly, for cell line xenografts, endurance exercise slowed the growth of 4T1 breast cancer cells implanted in the mammary fat pad [32], while another study, also using of endurance exercise training, reported differential sensitivity of three breast cancer cell lines, from 0.5-fold smaller (E0771 cells), 2-fold larger (C3(1)SV40Tag-p16-luc cells) and no change (4TO7 cells) compared to controls, under exactly the same experimental conditions [33]. This variation in response to exercise observed in murine models is not unique to breast and prostate cancer, and is also observed in preclinical models of colon, lung and skin cancer, where the pathophysiological effects of exercise on tumorigenesis are favorable but inconsistent (see review [30]). This is not surprising given the variation in mouse models studied to date, including genetic drivers for transgenic mice, subcutaneous or orthotopic grafting sites for xenograft models, and varying growth rates of mouse tumors *vs.* cell lines,

altogether providing unique genetic and microenvironmental influences on tumor growth. This, coupled with the multiple exercise protocols employed, likely underpins the discrepancy reported in the literature to date. Hence, based on this wide variance reported in mice, it is anticipated that exercise effects on tumor progression in humans is also likely to be heterogeneous.

Altered substrate metabolism is a hallmark of cancer and is documented to occur in prostate cancer, and exercise training is known to independently modulate substrate metabolism in multiple tissues including skeletal muscle and non-contracting tissues such as adipose tissue and liver [21]. Therefore, in this study, we assessed glucose, glutamine and fatty acid metabolism in control and exercise trained *Pten*$^{-/-}$ mice. Consistent with the lack of observable anti-tumorigenic effects of exercise, we did not detect any significant changes in substrate uptake, oxidation or incorporation into lipids. To our knowledge, this is the first direct assessment of substrate metabolism in prostate cancer after exercise training and despite the lack of significant changes, future studies might instead focus on the effects of exercise on other metabolic pathways and the production / accumulation of oncometabolites in prostate cancer.

Whilst there is limited pre-clinical, and no clinical, data to support the notion that exercise training improves prostate cancer prognosis in men with prostate cancer, there is convincing evidence that men with advanced prostate cancer benefit greatly from exercise regimes, particularly in terms of improved quality of life for men receiving ADT. For example, a 20-week program of resistance training led to significant improvements in muscle strength, walking ability, physical function, balance and cardiorespiratory fitness [34]. In response to endurance training, ADT-treated prostate cancer patients exhibited improved insulin sensitivity and reductions in fat mass and

body weight [35]. Similarly, a 12-month program of aerobic resistance training or impact loading resistance training successfully reduced fatigue and enhanced vitality in men with prostate cancer receiving ADT [36]. While these benefits are both expected and substantial, and exercise clearly has a major role in maintaining optimal care for prostate cancer patients undergoing hormone treatment, the effects on overall survival are not known.

One of the major barriers to regular endurance-based exercise is motivation. Recent evidence indicates that HIIT is more sustainable in the general population and induces metabolic and cardiovascular health benefits that are equivalent to, or in some cases, better traditional endurance training programs [37-39]. While it is conceivable that HIIT might be an effective intervention for prostate cancer, the evidence for this is limited [40]. While our data demonstrated that HIIT training was ineffective at reducing cancer progression in *Pten*$^{-/-}$ mice, HIIT may still be an attractive exercise type for patients preferring a short interval program as a component of their clinical care.

Conclusions

These pre-clinical data do not provide evidence to support the use of exercise therapy to reduce the early stages of tumor progression in prostate cancer associated with *Pten*$^{-/-}$ deletion. However, it is important to note that physical activity provides numerous health benefits and should still be considered for clinical oncology practice to optimize patient outcomes in men with prostate cancer.

Declaration of Interest: The authors declare that they have no competing financial interests.

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Figures

Figure 1**: Efficacy of exercise training in *Pten*$^{-/-}$ mice.** (A) Schematic of the study design. (B) Body weight over the duration of the experiment in control, endurance and high-intensity interval training (HIIT) mice. Data represented as mean ± SEM. Pre-training and post-training tests showing (C) endurance exercise capacity and (D) maximum running velocity in control, endurance and HIIT groups. Data represented as mean, with pre- and post-trained mice denoted by adjoining line. Control: n=10; Endurance: n=9; HIIT: n=6. Data are presented as means ± SEM (panel B) or individual data points and means (panels C-D). Data analyzed by two-way ANOVA for body weight and one-way ANOVA for exercise capacity and running velocity.

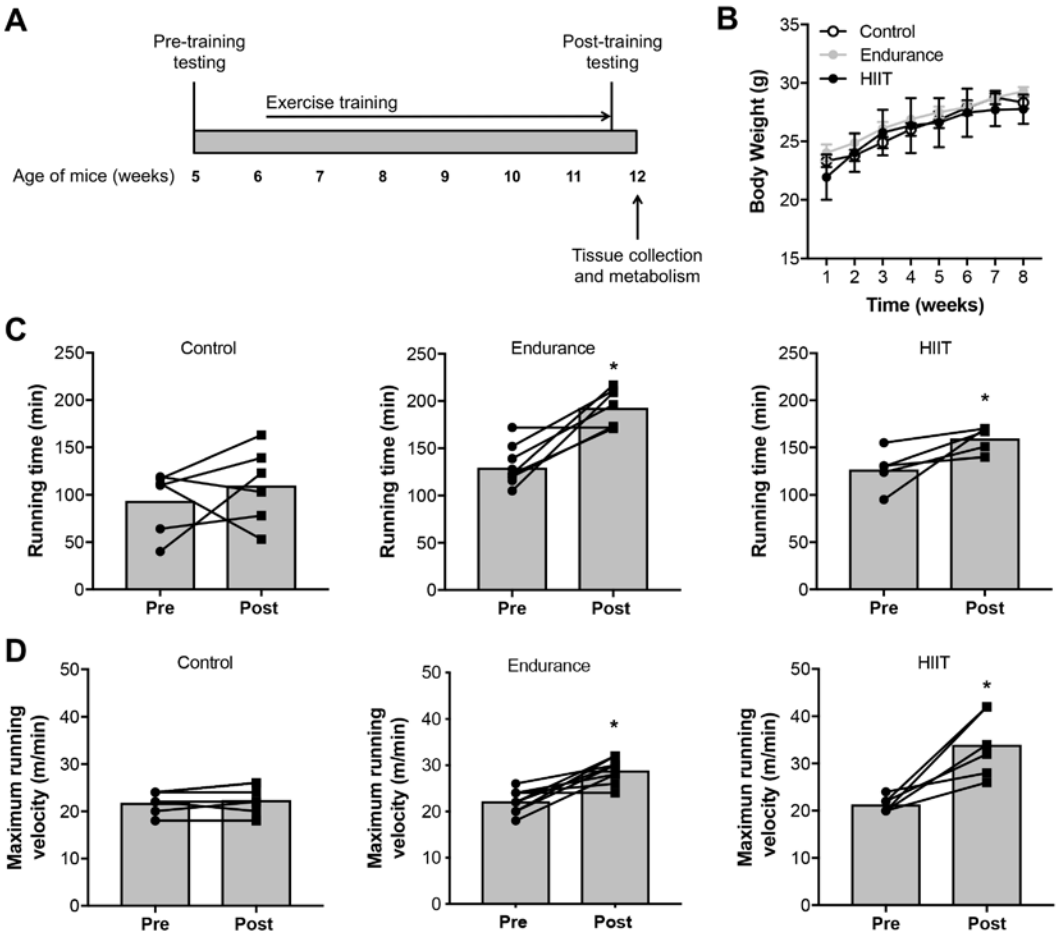

Figure 1

Figure 2**: Effect of exercise on prostate cancer progression.** (A) Prostate weight of combined lobes and (B) prostate weight of individual lobes including anterior prostate (AP), dorsal prostate (DP), ventral prostate (VP) and lateral prostate lobes (LP) in control, endurance and HIIT groups. (C) Proportion of prostate pathology graded as, low-grade prostatic intraepithelial neoplasia (LG-PIN), high-grade prostatic intraepithelial neoplasia (HG-PIN), or non-invasive carcinoma *in situ* (NI-CIS) in control, endurance and HIIT mice. Representative H&E images shown in right hand panels; scale represents 50μm. (D) Proliferation assessed by immunostaining for ki-67 in control, endurance and HIIT mice. Representative ki-67 images shown in right hand panels; scale represents 100μm. Control: n=10; Endurance: n=9; HIIT: n=6. Data are presented as means ± SEM and analyzed by one-way ANOVA. *P<0.05 *vs.* untrained mice and endurance trained mice.

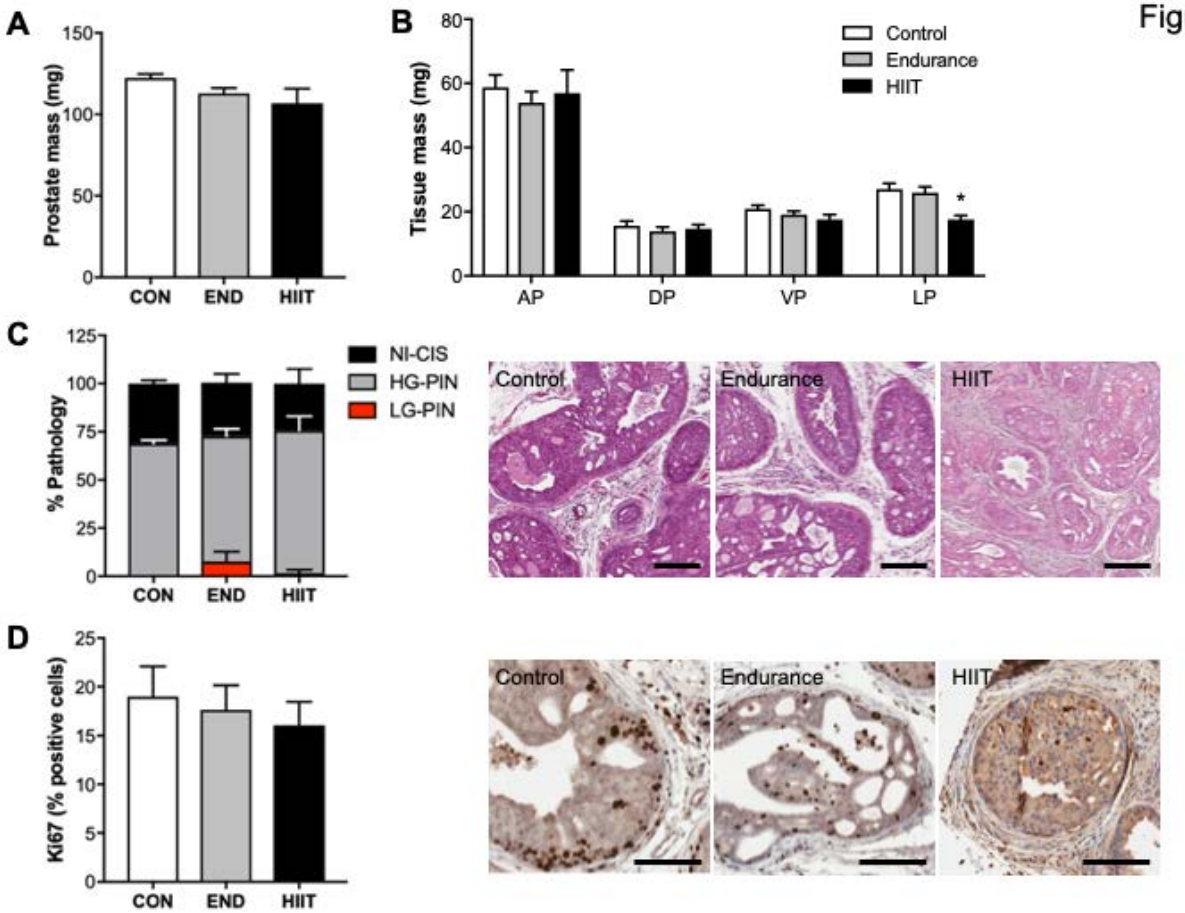

Figure 2

Figure 3**: Effect of exercise on glucose and glutamine metabolism.** (A-C) Glucose metabolism including (A) glucose uptake, (B) glucose oxidation and (C) *de novo* lipogenesis from glucose; Control: n=10; Endurance: n=9; HIIT: n=5. (D-E) Glutamine metabolism including (D) glutamine oxidation and (E) *de novo* lipogenesis from glutamine; Control: n=7-8; Endurance: n=5-8; HIIT: n=4. Data are presented as means ± SEM and were analyzed by one-way ANOVA.

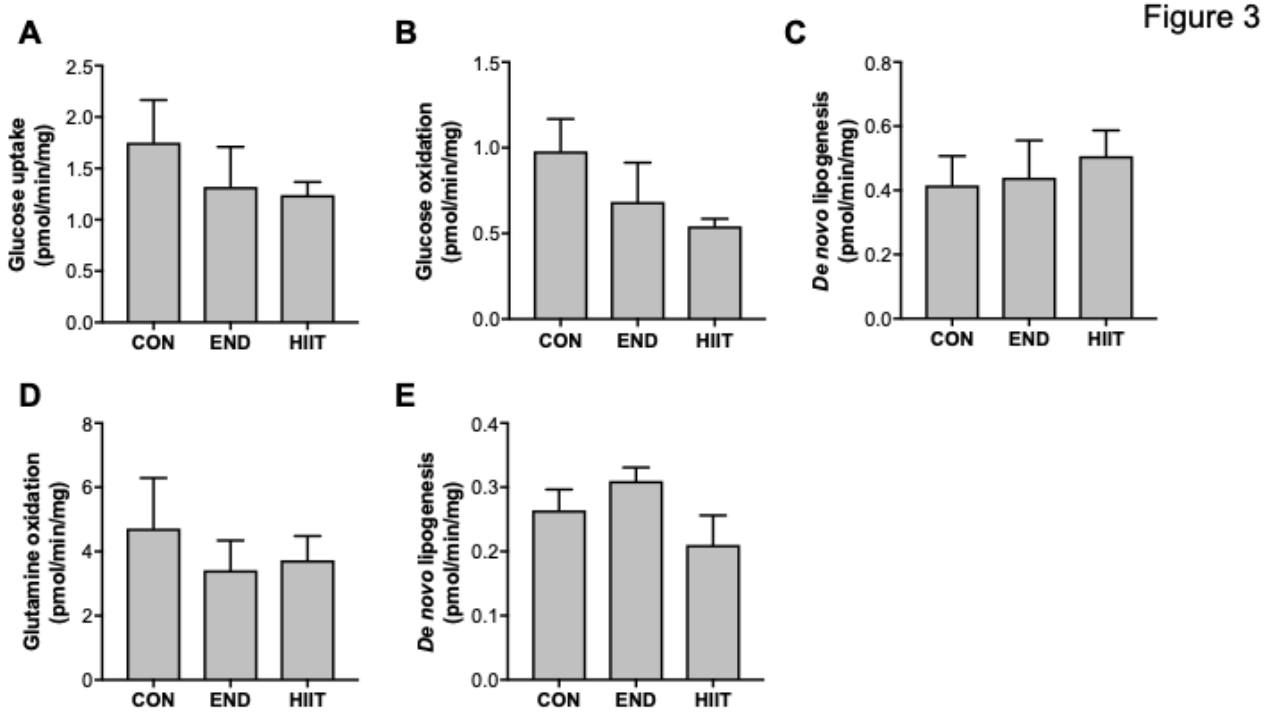

Figure 4**: Effect of exercise on fatty acid metabolism.** (A) Fatty acid uptake, (B) fatty acid oxidation, and (C) fatty acid storage into various lipids. Chol, cholesterol; Cer, ceramide; PL, phospholipid; DAG, diacylglycerol; TAG, triacylglycerol. Control: n=10; Endurance: n=7; HIIT: n=6. Data are presented as means ± SEM and were analyzed by one-way ANOVA.

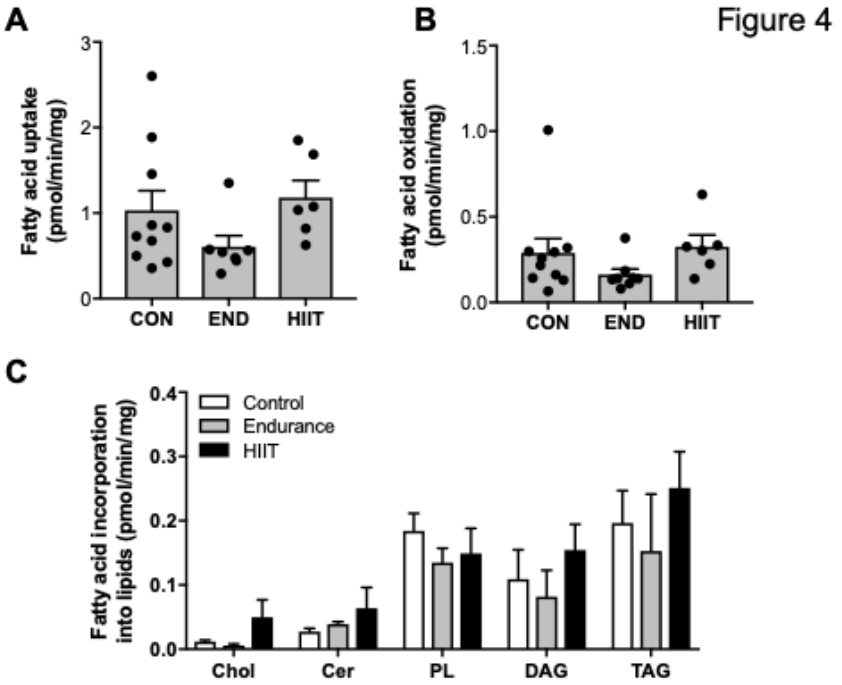

Table 1. Body mass, fat mass and blood chemistry in sedentary and exercise trained *Pten*$^{-/-}$ mice.

	Control	**Endurance**	**HIIT**
Body mass (g)	28.5 ± 0.4	29.1 ± 0.3	27.7 ± 0.8
Epididymal fat mass (mg)	443 ± 59	259 ± 26	577 ± 172
Plasma NEFA (mmol/L)	0.42 ± 0.04	0.46 ± 0.08	0.49 ± 0.08
Plasma TG (mmol/L)	1.55 ± 0.26	1.39 ± 0.18	1.99 ± 0.14
Plasma Insulin (pmol/L)	139 ± 30	135 ± 37	166 ± 33

Data represented as mean ± SEM. Body mass and epididymal fat mass presented for Control: n=10; Endurance: n=9; HIIT: n=6. Plasma measurements included where blood was available including Control: n=8; Endurance: n=8; HIIT: n=5. Data analyzed by one-way ANOVA.