1 Voluntary exercise does not improve muscular properties or functional capacity
2 during C26-induced cancer cachexia in mice

3
4 Charlotte Hiroux^{1*}, Sebastiaan Dalle^{1*}, Katrien Koppo¹, Peter Hespel¹

5
6 ¹Exercise Physiology Research Group, Department of Movement Sciences, KU Leuven, Leuven,
7 Belgium

8 Tervuursevest 101 box 1500, 3001 Leuven, Belgium

9 * Contributed equally to the work

10 ORCIDs:

11 CH: 0000-0001-8345-0119

12 SD: 0000-0001-5734-3993

13 KK: 0000-0002-6022-1097

14 PH: 0000-0003-1283-2229

15 Corresponding author:

16 Peter Hespel

17 Address: Exercise Physiology Research Group, Department of Movement Sciences, KU
18 Leuven, Tervuursevest 101 box 1500, 3001 Heverlee, Belgium

19 E-mail: peter.hespel@kuleuven.be

20 Tel: +32 16 32 90 91

ABSTRACT

Exercise training is considered as a potential intervention to counteract muscle degeneration in cancer cachexia. However, evidence to support such intervention is equivocal. Therefore, we investigated the effect of exercise training, i.e. voluntary wheel running, on muscle wasting, functional capacity, fiber type composition and vascularization during experimental cancer cachexia in mice. Balb/c mice were injected with PBS (CON) or C26 colon carcinoma cells to induce cancer cachexia (C26). Mice had free access to a running wheel in their home cage (CON_{EX} and C26_{EX}, n=8-9) or were sedentary (CON_S and C26_S, n=8-9). Mice were sacrificed 18 days upon tumor cell injection. Immunohistochemical analyses were performed on m. gastrocnemius and quadriceps, and *ex vivo* contractile properties were assessed in m. soleus and extensor digitorum longus (EDL). Compared with CON, C26 mice exhibited body weight loss (~20%), muscle atrophy (~25%), reduced grip strength (~25%), and lower twitch and tetanic force (~20%) production in EDL but not in m. soleus. Furthermore, muscle of C26 mice were characterized by a slow-to-fast fiber type shift (type IIx fibers: +57%) and increased capillary density (~30%). In C26 mice, wheel running affected neither body weight loss, nor muscle atrophy or functional capacity, nor inhibited tumor growth. However, wheel running induced a type IIb to type IIa fiber shift in m. quadriceps from both CON and C26, but not in m. gastrocnemius. Wheel running does not exacerbate muscular degeneration in cachexic mice, but, when voluntary, is insufficient to improve the muscle phenotype.

Key words: cancer cachexia, voluntary wheel running, muscle atrophy, muscle functionality, fiber type composition, capillary density

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INTRODUCTION

Cancer is a major cause of death worldwide (Bray et al. 2018). Depending on tumor type, 50 to 80% of cancer patients are afflicted by cachexia (Argilés et al. 2014), a multifactorial syndrome characterized by an ongoing loss of skeletal muscle mass, with or without the loss of fat mass (Fearon et al. 2011). This degenerative process cannot be fully reversed by conventional nutritional support (Fearon et al. 2011). Progressive muscle wasting leads to weakness, fatigue and decreased functional capacity which impairs patients' quality of life (Argilés et al. 2014) (Fearon et al. 2012). Moreover, the cachexia syndrome blunts the efficacy of cancer therapy and is estimated to account for ~20% of cancer deaths (Argilés et al. 2014). Despite detrimental clinical outcomes, the pathophysiological mechanisms underlying cachexia are not yet fully understood and pertinent treatments are still lacking (Aversa et al. 2017). Since the cachexia syndrome is multifactorial, multimodal interventions are needed in the therapeutic management of cachexia.

Exercise training, complementary to pharmacological and nutritional approaches, is often proposed to be integrated in the multimodal treatment of cachexia (Argilés et al. 2012; Gould et al. 2013; Aversa et al. 2017). In healthy conditions, physical activity and training can increase muscle mass, enhance muscular strength or endurance, and improve overall cardiovascular fitness (Hawley et al. 2014). Whereas in cancer patients the role of physical activity has been a matter of debate. A Cochrane systematic review from 2014 concluded that there is insufficient evidence to determine the safety and effectiveness of exercise in patients with cancer cachexia (Grande et al. 2014). But more recent reports considered exercise to be feasible, safe (Cave et al. 2018; Grote et al. 2018; Wiskemann et al. 2019) and even effective to improve muscular adaptations in cancer patients undergoing adjuvant therapy (Padilha et al. 2017; Mijwel et al. 2018; Češeko et al. 2020). In cancer patients, exercise training was found to reduce fatigue and improve physical function and quality of life (Heywood et al. 2018). Furthermore, a systematic review concluded that both aerobic and resistance type exercise can improve muscle strength in cancer patients during treatment, compared to usual care alone (Stene et al. 2013), especially when physical activity is started in the pre-cachectic stage rather than during the cachexia (Antoun and Raynard 2018). Still, there is a need for more studies to confirm the beneficial muscular adaptations in response to exercise, both at a mechanistic and functional level. To this background, cancer cachexia models in rodents are a promising tool to investigate the effects of exercise on i) the isolated muscular functional capacity and ii) myocellular adaptations. Unfortunately, results from studies that have looked at the therapeutic potential of exercise training in rodent models of experimental cancer cachexia (Pin et al. 2015; Coletti et al. 2016; das Neves et al. 2016; Jee et al. 2016; Pigna et al. 2016; Padrão et al. 2017; Alves et al. 2018; Tatebayashi et al. 2018; Ballarò et al. 2019b; Tanaka et al. 2019) remain equivocal. But discrepancies are at least partly due to different cachexia models and exercise interventions used.

To date, preclinical studies have primarily focused on the molecular mechanisms involved in muscular adaptations to exercise training in cancer cachexia. However, only a small number of studies have addressed cellular and functional adaptations, such as muscle fiber type composition, capillary density and contractile properties (Pigna et al. 2016) Tanaka et al. 2019; Alves et al. 2018; das Neves et al. 2016). Endurance training induces a fast-to-slow fiber type shift (Schiaffino and Reggiani 2011) and increases capillary density (Hawley et al. 2014; Egan and Zierath 2013), thereby improving the oxidative capacity

and eventually endurance exercise tolerance. However, the catabolic events inherent to cancer cachexia *per se* might also interfere with the muscular adaptations to exercise, e.g. fiber type composition and vascularization. Indeed, it was suggested that cancer cachexia *per se* might induce a slow-to-fast muscle fiber type transition in mice (Diffie et al. 2002; Roberts et al. 2013). Furthermore, to our knowledge, only one study has investigated the effect of cachexia and exercise on muscle vascularization (Tanaka et al. 2019). They concluded that low-intensity treadmill running prevented the cachexia-induced decrease in the capillary-to-fiber ratio in rats injected with AH130 Yoshida hepatoma cells (Tanaka et al. 2019). Also in breast cancer patients, exercise training prevented the loss in muscle capillarisation during chemotherapy, compared to patients undergoing the usual care (Mijwel et al. 2018). Altogether, there are indications that cancer cachexia interacts with the muscular adaptations to exercise training, but this remains to be further confirmed.

Therefore, the aim of the current study is to investigate the effect of exercise training in the form of voluntary wheel running on muscle fiber type composition, vascularization and functional capacity during cancer cachexia development in C26 mice. For this purpose mice were randomly assigned to four experimental groups: healthy control mice (CON_S), healthy mice performing voluntary wheel running (CON_{EX}), C26 mice (C26_S) and C26 mice performing voluntary wheel running (C26_{EX}).

METHODS

Mice

Male BALB/c mice, 7-8 weeks old, were randomized into four experimental groups: i) healthy control mice remaining sedentary (CON_S, n=9), ii) healthy control mice performing voluntary wheel running (CON_{EX}, n=9), iii) C26 mice remaining sedentary (C26_S, n=8) and iv) C26 mice performing voluntary wheel running (C26_{EX}, n=8). Since C26_S and C26_{EX} were pair-matched, death of one resulted in the exclusion of the other pair-matched mouse from the study. C26 mice were subcutaneously inoculated in the upper back with 5x10⁵ murine colon carcinoma C26 cells dissolved in 100μl phosphate-buffered saline (PBS). Control mice were injected with 100μl PBS. The C26 cell line was cultured in DMEM (Thermo Fisher Scientific, 41965-062) supplemented with 10% fetal bovine serum (Life Technologies, 10270-106), 1% glutamine (Sigma, G7513) and 1% penicillin and streptomycin (Sigma, P4333). Animals were sacrificed 18 days after PBS or tumor cell injection (Figure 1). All mice were housed individually (Type III macrolon cages) in a controlled environment with 12:12 h light-dark cycles and free access to food and water. Food intake was recorded by weighing the food pellets every 24 h until day 17. All procedures were approved by the Animal Ethics Committee of KU Leuven, Belgium (P187-2015). Throughout the manuscript *pretest* refers to the data obtained before tumor cell injection, whereas *posttest* refers to measurements at the end of the experimentally-induced cancer cachexia period, i.e. the day before (day 17) or on sacrifice day (day 18).

Exercise protocol

Mice in the exercise groups (CON_{EX} and C26_{EX}) were housed individually in cages equipped with a wheel for rodents (ø 12 cm) starting from the day before C26/PBS injection (day -1) to allow for a 24 h familiarization period. Mice had free access to the wheel until 24h before sacrifice (day 17) to reduce

the confounding effects of acute exercise on the measurements. Each wheel was supplied with a cycle computer (2Cycle, 81488, Sint Annaparochie, The Netherlands) to record running activity.

Grip strength

Grip strength testing was performed during the pre- and posttest. All mice were familiarized with the grip strength device (BioSeb, BIO-GS3, Chaville, France) and protocol four days before injection. Mice were held by the tail and were allowed to grasp the pull bar with their forelimbs or the grid with all four limbs and then pulled backwards in the horizontal plane. Mice performed 5 trials with 3 min rest intervals in between. The best three attempts were averaged and used for analysis.

Ex vivo muscle contractile properties

After mice were anaesthetized (Ketamine and 2% Xylazine), m. extensor digitorum longus (EDL) and m. soleus (SOL) from both limbs were carefully dissected and strings were attached to the proximal and distal tendon of the muscle. Each muscle was mounted vertically with one tendon attached to a force transducer and stimulated between platinum electrodes. The muscle was continuously immersed in Krebs buffer (118 mM NaCl, 25 mM NaHCO₃, 2.4 mM MgSO₄*7H₂O, 3.4 mM CaCl₂*2H₂O and 5 mM glucose) at a temperature of 37°C and gassed with a mixture of 95% O₂ and 5% CO₂. First, the optimal muscle length (L₀) was determined by tetanic contractions. To investigate twitch characteristics, EDL and SOL were stimulated 3 times at 5 Hz with 1 min rest intervals. For the tetanic characteristics, muscles were stimulated 3 times during 250 ms at 120 Hz (EDL) and during 500 ms at 80 Hz (SOL) with 2 min rest intervals in between. The maximal specific tension, i.e. the force corrected for the estimated cross-sectional area (CSA), was calculated for twitch and tetanic contractions. CSA was estimated by dividing the wet muscle mass by the product of muscle length (L₀) and 1.06 g/cm³, the density of mammalian skeletal muscle. Due to technical errors, the EDL results of one mouse in C26_{EX} were lost.

Spontaneous physical activity

Spontaneous physical activity was assessed during 24 hours at pretest, i.e. 24 h before familiarization to the wheel (day -2 till day -1), and at posttest, i.e. 24 h before removing the wheels (day 16 till day 17). The activity was assessed by video analyses in a subset of mice (n=5-6 per experimental group) residing in their home cage and without interfering with normal behavior, as we have previously described (Poffé et al. 2018).

Muscle sample preparation

M. gastrocnemius (GA) and m. quadriceps (QU) were isolated, weighed and used for histological analyses. The muscles were embedded in tissue freezing medium (Leica Biosystems, 14020108926), frozen in liquid nitrogen-cooled isopentane and stored at -80°C. Transverse sections of 7 µm thickness were obtained using a cryostat (Leica Biosystems, CM1850, Germany).

MHC fiber type staining and cross-sectional area

Frozen cryosections of the mid-belly region of GA and QU muscles were used to stain the different isoforms of myosin heavy chain (MHC) proteins, as previously described (Bloemberg and Quadrilatero 2012). Briefly, cryosections were air-dried at room temperature and placed in blocking solution (1%

BSA in PBS) for 2 h. Afterwards, samples were incubated overnight at 4°C with a cocktail of primary antibodies containing BA-F8 (type I fibers, 1:400, DSHB, University of Iowa), SC-71 (type IIa, 1:100, DSHB, University of Iowa), BF-F3 (type IIb, 1:300, DSHB, University of Iowa) and laminin (1:500, Sigma, L9393) in 0.5% BSA. Sections were then incubated for 1 h at room temperature with a cocktail of secondary antibodies containing Alexa fluor 488 goat anti-mouse IgG2b (type I, 1:300, Life Technologies, A21141), Alexa fluor 350 goat anti-mouse IgG1 (type IIa, 1:300, Life Technologies, A21120), Alexa fluor 594 goat anti-mouse IgM (type IIb, 1:300, Life Technologies, A21044) and Alexa fluor 488 donkey anti-rabbit IgG (sarcolemma, 1:600, Life Technologies, A21206) in 0.5% BSA. Images from the whole cross-section (10x) were acquired with Nikon Eclipse E1000 microscope (Nikon, Boerhavedorp, Germany) using the NIS Elements software (Nikon Instruments Europe BV, Amsterdam, The Netherlands). The entire cryosection was reconstituted using Microsoft Image Composite Editor (Microsoft, Cambridge, USA). Type I, IIa, IIx and IIb fibers in the entire cross-section were counted using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). Overall, approximately 5960 fibers per cross-section were analyzed in GA versus 2460 fibers in QU. To determine fiber CSA, 30 randomly chosen fibers of each fiber type were manually outlined in each cryosection using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). Preliminary analysis indicated that 30 fibers per fiber type per section yields a representative measurement of the fiber CSA. All analyses were done by investigators who were blinded to the experimental conditions. Because type I fibers represented less than 1.5% and 0.5% of fibers in GA and QU, respectively, they were not considered in the analyses.

Capillary staining

Muscle cryosections were air dried and incubated in blocking solution (1% BSA in PBS) at room temperature for 2 h. Samples were then incubated with isolectin conjugated with Alexa Fluor 568 (capillaries, 1:500, Life Technologies, I21412) and wheat germ agglutinin conjugated with Alexa Fluor 350 (sarcolemma, 1:100, Life Technologies, W11263) in PBLEC for 1 h. Capillary density was quantified by manually counting of isolectin-positive signals on 5-6 images from each cryosection (20x) with a Nikon Eclipse E1000 microscope (Nikon, Boerhavedorp, Germany) using the NIS Elements software (Nikon Instruments Europe BV, Amsterdam, The Netherlands). Analyses were performed by investigators who were blinded to the experimental groups.

Succinate dehydrogenase staining

Muscle cryosections were air dried and incubated with 25% V/V Tris-HCl (50mM, pH7.4), 25% V/V Na-succinate (0.2M), 5% V/V MgCl₂*6H₂O (50mM) and 45% V/V nitro blue tetrazolium chloride (1.223mM) for 1 h at 37 °C in a humid chamber. Images from the whole cross-section (10x) of n=6/condition were acquired with Nikon Eclipse E1000 microscope (Nikon, Boerhavedorp, Germany) using the NIS Elements software (Nikon Instruments Europe BV, Amsterdam, The Netherlands). The entire cryosection was reconstituted using Microsoft Image Composite Editor (Microsoft, Cambridge, USA). The blue-purple color intensity was quantified to assess the succinate dehydrogenase activity in at least n=50 oxidative fibers per cryosection using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistics

All results are expressed as mean $\pm$ SEM. To identify significant differences between the experimental conditions, a two-way ANOVA (EX vs. S & C26 vs. CON) followed by Bonferroni post-hoc test. Differences in body weight, spontaneous physical activity and grip strength were analyzed using a three-way repeated measures ANOVA (EX vs. S & C26 vs. CON & pretest vs. posttest) followed by Bonferroni post-hoc test. Differences in running distance were analyzed using a two-way repeated measures ANOVA (C26 vs. CON & pretest vs. posttest) followed by Bonferroni post-hoc test. Differences in muscle weight were analyzed with a two-way repeated measures ANOVA (EX vs. S & C26 vs. CON) followed by a Bonferroni post-hoc test. Differences in tumor growth were analyzed using a Student's t-test. All statistical analyses were performed using IBM SPSS Statistics 25 (IBM, New York, USA). Statistical significance was defined as $p < 0.05$.

RESULTS

C26 mice performed less wheel running activity and spontaneous home cage physical activity

Overall, C26_{EX} were less active in wheel running than CON_{EX} (CON_{EX}: 12.4 ± 1.8 km/day; C26_{EX}: 6.7 ± 1.0 km/day, $p < 0.05$) (*Figure 2*). In CON_{EX}, daily running distance increased from 4.6 ± 1.5 km on day 1 to 16.9 ± 2.2 km on day 17. Conversely, in C26_{EX}, daily running distance increased from 4.1 ± 1.3 km on day 1 (n.s. vs. CON_{EX}) to 8.9 ± 1.6 km on day 4 (n.s. vs. CON_{EX}) but thereafter declined to 4.4 ± 1.2 km on day 17 ($p < 0.05$ vs. CON_{EX}) (*Figure 2*). Running speed was not significantly different between the groups at any time. Spontaneous locomotor physical activity in the home cage, other than wheel running, was at pretest on average ~ 825 m (509 m – 1304 m) and was similar between the groups. At posttest, C26 mice performed less spontaneous physical activity than CON (CON: 682 ± 83 m; C26: 356 ± 46 m, $p < 0.05$). Wheel running activity *per se* did not alter spontaneous activity level in CON nor in C26. Overall, daily food intake was on average 5.8 ± 2.2 g and similar between C26 and CON ($p > 0.05$). However, wheel running increased daily energy intake by $\sim 20\%$, both in CON and C26 mice ($p < 0.05$).

C26 mice developed the cachectic phenotype which was not reversed by voluntary wheel running

Body weight (BW) was similar between the groups at pretest. BW declined from day 8 till the end of the experiment in C26 but not in CON ($p < 0.05$) (*Figure 3*). At posttest, BW on average was $\sim 20\%$ lower in C26 than in CON ($p < 0.05$). Voluntary wheel running affected BW neither in CON nor in C26 (*Figure 3*). In line, carcass weight at posttest, i.e. BW minus tumor mass, was $\sim 20\%$ lower in C26 than in CON ($p < 0.05$) and this was not affected by wheel running ($p > 0.05$) (data not shown). At posttest, muscle mass was consistently reduced in C26 compared to CON (GA: -27% , QU: -35% , EDL: -27% , SOL: -15% , $p < 0.05$) (*Figure 4*). Also, muscle mass to BW ratio was lower in C26 than in CON ($p < 0.05$), except for SOL (data not shown). Voluntary wheel running did not attenuate muscle wasting in C26. Compared with CON, total body fat mass at posttest was reduced by $\sim 56\%$ in C26 mice ($p < 0.05$). Fat mass loss appeared in both visceral fat (-37% , $p < 0.05$), epididymal fat (-88% , $p < 0.05$) and brown adipose tissue (-63% , $p < 0.05$). Irrespective of the experimental condition, wheel running reduced total fat mass by 21% ($p < 0.05$), of which visceral fat mass by 17% ($p < 0.05$) and epididymal fat pads by 34% ($p < 0.05$) (*Table 1*). Spleen mass was substantially higher in C26 mice than in CON ($+151\%$, $p < 0.05$), whilst cardiac mass was reduced (-13% , $p < 0.05$). Liver mass was unaffected by C26. Voluntary wheel running

affected neither spleen, nor heart nor liver mass (*Table 1*). Tumor weight was similar between C26_s (476 ± 59 g) and C26_{EX} (475 ± 34 g).

Voluntary wheel running did not prevent the loss of muscular functional capacity in C26 mice

Muscle function was assessed by *in vivo* grip strength and by muscle force outputs during electrically-induced *ex vivo* muscle contractions. At pretest, grip strength was similar between the experimental conditions. From pre- to posttest, both absolute (g) and relative ($\text{g}\cdot\text{g}^{-1}$ BW) forelimb grip strength increased in CON (+17% and +14% respectively, $p<0.05$) while it decreased in C26 (-25% and -8% respectively, $p<0.05$). Thus, at posttest, absolute and relative forelimb grip strength were ~30% and ~15% lower in C26 than in CON ($p<0.05$). Accordingly, the 4-limb absolute grip strength declined from pre- to posttest in C26 (-20%, $p<0.05$) resulting in ~20% lower force than in CON ($p<0.05$). Nonetheless, relative 4-limb grip strength was similar between C26 and CON. Wheel running did not affect grip strength in CON nor in C26 (*Table 2*). Compared with CON, *ex vivo* twitch and tetanus specific tensions in EDL were lower in C26 (-21% and -17% respectively, $p<0.05$) (*Figure 5 A-B*). In contrast, twitch and tetanic specific tensions in SOL were similar between C26 and CON. In SOL, wheel running increased twitch and tetanus tensions by respectively ~50% and ~40% in CON ($p<0.05$) but not in C26 (*Figure 5 C-D*).

Voluntary wheel running induced a fast-to-slow fiber type shift while cachexia increased muscle capillary density and mitochondrial capacity

Total fiber number in GA and QU was similar between the experimental conditions ($p>0.05$). Compared with CON, and irrespective of wheel running, % type IIx fiber CSA in GA muscle was increased by ~57% in C26 mice ($p<0.05$). Conversely, in QU muscle of both CON and C26 mice, wheel running increased the proportion of type IIa fiber CSA about 2-fold ($p<0.05$), whilst type IIb fiber CSA fraction was slightly reduced (-9%, $p<0.05$). Relative fiber number changes paralleled changes in proportional CSA's (*Table 3*). Both in GA and QU, mean CSA of type IIa, IIx and IIb fibers was 20-40% lower in C26 than in CON ($p<0.05$). When type II fiber subtypes were taken together, their CSAs were on average ~30% lower in both GA and QU muscles of C26 mice than in CON ($p<0.05$). Compared to sedentary mice, wheel running tended to increase (~13%) type IIb fiber CSA in GA, but not in QU, both in CON and in C26 ($p=0.080$). Furthermore, compared with CON, capillary density was ~30% higher in both GA and QU muscles of C26 mice. However, voluntary wheel running did not affect capillary density in GA or in QU (*Figure 6*). Wheel running increased the activity of the mitochondrial enzyme succinate dehydrogenase in QU of both CON (+25%) and C26 (+9%), but this was not significant ($p=0.158$). However, the cachexia phenotype significantly increased the mitochondrial capacity in QU of C26 vs. CON (+63%; $p<0.001$; Suppl. Fig. 3).

DISCUSSION

It is believed that physical activity may be a useful adjuvant therapy to counteract muscular degeneration in cancer cachexia (Argilés et al. 2012; Aversa et al. 2017). Yet, strong and unambiguous evidence to support such beneficial adaptations to exercise are still lacking (Grande et al. 2015). To date, a series of preclinical studies yielded equivocal results and mainly focused on molecular changes

occurring in muscular degeneration during cachexia(das Neves et al. 2016; Pigna et al. 2016; Alves et al. 2018; Tanaka et al. 2019). However, structural and functional adaptations to exercise remain poorly explored in cachexic muscle. Therefore, the current study addressed the effects of exercise training via voluntary wheel running on muscle fiber type composition, CSA, vascularization, and functional capacity during experimental cancer cachexia in C26 mice.

This particular cancer cachexia murine model was applied because it is believed to be a valid preclinical model of cancer-induced muscle wasting(Aulino et al. 2010; Murphy et al. 2012; Talbert et al. 2014). In line with earlier findings(Aulino et al. 2010; Murphy et al. 2012), the current experimental conditions induced severe functional and muscular degeneration. However, consistent exercise via voluntary wheel running impacted neither muscle mass wasting or functional deterioration, nor tumor growth. Furthermore, we suggest that cancer cachexia attenuated some muscular adaptations to exercise. The *ex vivo* SOL muscle strength increased in response to voluntary wheel running in healthy but not in C26 mice, despite similar changes in muscle fiber type phenotype and body composition.

The primary aim of this study was to investigate the impact of exercise in the form of voluntary wheel running, during cancer cachexia development. As expected, wheel running activity gradually increased in CON(Coletti et al. 2013), whereas in C26, running activity leveled off by day 4 and markedly dropped afterwards. Remarkably, even at late cachexia stage when body weight had dropped by ~20%, C26 mice were still able to perform wheel running activity.

In line with previous data(Aulino et al. 2010; Murphy et al. 2012), cachexia resulted in explicit muscular atrophy as muscle masses on average decreased by ~26%. Yet, responses were heterogeneous between muscles. The atrophy was about 2-fold greater in EDL (~27%), predominantly composed of type II fibers, than in SOL (~15%) which contains high fraction of type I fibers. This confirms that cachexia negatively impacts glycolytic muscles more than oxidative muscles(Acharyya et al. 2005; Li et al. 2007; Ciciliot et al. 2013; Wang and Pessin 2013).

Whereas we observed no protective effect of voluntary wheel running on fiber type atrophy in cachexic mice, Pin et al. (2015) reported that exercise training might spare oxidative fibers from atrophying and thereby counteract the oxidative-to-glycolytic shift(Pin et al. 2015). However, in their study, mice were enrolled in 5 weekly training sessions (45 min at 14 m·min⁻¹) from 6 weeks prior to tumor injection while being treated with erythropoietin (Pin et al. 2015). It can be questioned whether these conditions mimic real-life settings. Underlying mechanism(s) to explain the fiber type shift in cachexia are not yet fully elucidated. However, it was suggested that a higher expression of peroxisome proliferator-activated receptor gamma coactivator 1 α in oxidative fibers protect against atrophy in cancer cachexia(Wang and Pessin 2013) whereas FoXO and NF- κ B signaling tends to primarily affect fast-twitch glycolytic fibers(Ciciliot et al. 2013; Wang and Pessin 2013).

A higher degree of atrophy in EDL than in SOL also translated into functional impairment in the former but not in the latter. However, the extent to which cachexia impaired absolute twitch and tetanic force development in EDL (minus ~38%) exceeded the degree of atrophy (minus ~27%). This indicates that the reduced force generating capacity might also be related to alterations in the dystrophin glycoprotein complex(Acharyya et al. 2005), mitochondrial dysfunctions(Antunes et al. 2014) and/or a reduction in the strongly bound cross-bridges(Toth et al. 2013). At a molecular level, it was suggested

that functional deficits in cachexia are related to muscle inflammatory signaling(Dalle et al. 2017; VanderVeen et al. 2018; Dalle and Koppo 2020). Interestingly, the SOL was resistant to cachexia-induced muscle functional impairments. Similar to differences in the fiber type-specific atrophy, it can be hypothesized that molecular processes affecting muscle quality occur earlier or are more pronounced in fast-twitch muscles (QU, GA) than in slow-twitch muscles (SOL). Importantly, consistent exercise did not counteract the aforementioned symptoms of muscular degeneration in any muscle.

To our knowledge, this is the first study that looks at the effect of cancer cachexia, alone or in conjunction with endurance exercise training, on both muscle fiber type distribution and capillary density. One earlier study found 10 days of low-intensity treadmill running to inhibit the decline in capillary-to-fiber ratio in AH130-induced cachexia in rats(Tanaka et al. 2019). In the current study, cancer cachexia *per se* increased the muscle capillary density in muscles with ~30%. The increase was largely, if not entirely due to smaller muscle fiber diameters, which even in the absence of *de novo* angiogenesis results in higher number of capillaries per surface area unit. Indeed, the relative increase in capillary density (~30%) was equivalent to the degree of muscle atrophy in both GA and QU. Still, the generated smaller diffusion distance might be considered as a beneficial muscular adaptation facilitating the delivery and diffusion of oxygen and therapeutic agents to muscle cells(Poole et al. 2013). Yet, this might also cause a higher exposure of muscle tissue to tumor-released pro-inflammatory factors(Acharyya et al. 2005).

Interestingly, we also observed that the cachexia phenotype increased the mitochondrial capacity in oxidative muscle fibers (+63%; $p<0.001$), irrespective of wheel running. The higher mitochondrial capacity coincided with lower physical activity (i.e. SPA and wheel running), and thus lower need for ATP generation, in the C26 mice vs. CON mice. Therefore, it is likely to assume that the cachexia-induced increased mitochondrial capacity reflects mitochondrial degeneration, typically observed in cancer cachexia and preclinical models(Julienne et al. 2012; Argilés et al. 2015). The absence of a training effect on vascularization and mitochondrial capacity in both healthy and cachectic muscles suggests that the duration and/or volume of the training intervention was possibly too low to significantly stimulate angiogenesis and mitochondrial adaptations(Landisch et al. 2008).

Cancer cachexia also impacted on the muscle fiber type composition. Our data confirm a fast-to-slow shift in muscle fibers due to cancer cachexia in the GA, i.e. minus ~5% of the fast type IIb fibers ($p>0.05$) and +5% of type IIx fibers ($p<0.05$) vs. CON. However, this was not confirmed in QU. Endurance exercise training elicited a type IIb-to-IIa fiber shift, both in healthy and cachectic QU. This indicates that even during the process of cachectic degeneration, muscles are responsive to exercise-induced adaptations. Surprisingly, these adaptations were entirely absent in GA. This explicit intermuscular diversity can at least partially be explained by differences in fiber type distribution, differential recruitment in concentric versus eccentric contractions during wheel running(Armstrong and Laughlin 1983), and/or divergent responsiveness to exercise stimuli and/or cancer development.

Findings regarding the effects of exercise on cancer cachexia-related muscular degeneration are equivocal. This is due to the use of different murine models submitted to different experimental conditions, e.g. different exercise interventions, different timing of the exercise interventions relative to the time of cancer induction, use of variable endpoints to assess muscular functional capacity in different

muscle types, and presence of adjuvant treatments. Furthermore, very similar studies showed that wheel running attenuates cachexia in female mice (Coletti et al. 2016; Pigna et al. 2016). This might be due to biological sex-differences that interfere with adaptations to cachexia and/or exercise, e.g. differences in food intake (Smarr et al. 2019), in baseline physical activity (Smarr et al. 2017) and in endocrine profile affecting systemic and muscular responses (Glenmark et al. 2004). Some (Coletti et al. 2016; Jee et al. 2016; Pigna et al. 2016; Padrão et al. 2017; Ballarò et al. 2019b, a; Tanaka et al. 2019; VanderVeen et al. 2020) but not all (Pin et al. 2015; das Neves et al. 2016; Padrão et al. 2017; Alves et al. 2018; Ballarò et al. 2019b, a) studies found small positive effects with either endurance training (Pin et al. 2015; Coletti et al. 2016; Jee et al. 2016; Pigna et al. 2016; Padrão et al. 2017; Ballarò et al. 2019b, a; Tanaka et al. 2019) or resistance training (das Neves et al. 2016) during cachexia progression.

A similar exercise intervention in C26 mice counteracted muscular atrophy in m. tibialis anterior (TA), but not in GA (Ballarò et al. 2019b). Indeed, muscular adaptations to wheel running are more pronounced in TA than in GA (Allen et al. 2001). Consequently, the limited amount of muscular adaptations to wheel running in this study can be attributed to the selection of SOL, GA, QU and EDL, but not TA, as target muscles. Nonetheless, one would expect at least some adaptations in these muscles, be it less pronounced than in TA. Another reason for the limited muscular adaptations might be the exercise stimulus. Training volume, duration and/or intensity might have been insufficient to generate significant muscular adaptations within the 2-3 week time window. In addition, training volume was even substantially lower in C26 mice than in CON, especially in the final stage of cancer development. However, this phenomenon reflects real-life conditions, in which cancer patients are likely to perform less exercise than healthy controls. Interestingly, cachexia *per se* did not reduce food intake, and wheel running similarly increased food intake in C26 and CON. Thus, the cancer-induced muscular degeneration seen in C26 mice was not due to inadequate energy intake, which is in line with previous data (Pigna et al. 2016; Tatebayashi et al. 2018; Diffie et al. 2002; Tanaka et al. 1990).

Tanaka et al. (2019) showed that low-intensity treadmill running can reduce muscle wasting in AH130-induced cachectic rats, whilst moderate-intensity exercise exaggerated cachexia development resulting in increased death incidence (Tanaka et al. 2019). In contrast, others demonstrated that high-intensity treadmill running may be more efficacious than moderate-intensity to prevent cancer-induced muscle wasting (Jee et al. 2016). Importantly, it was also shown that two weeks of treadmill running after tumor injection deteriorated muscle wasting in C26 mice (Pin et al. 2015). But, this detrimental effect was undone either by starting the exercise intervention 6 weeks prior to tumor injection, or by co-ingestion of erythropoietin to prevent anemia, which might impair exercise tolerance, indeed (Pin et al. 2015). One study also looked at the effects of motorized wheel running in C26 mice receiving chemotherapy, which probably yields a more relevant therapeutic context. Exercise training partially counteracted the loss of muscle mass and strength after ~4 weeks, but worsened survival in late stages of cachexia (Ballarò et al. 2019a). These results suggest that the exercise parameters may depend on tumor type and cachexia stage. To date, the current and earlier data obtained in murine cachexia models do not provide unambiguous support for exercise recommendations in cancer cachexia. Besides, well-controlled exercise intervention trials in cancer patients with cachexia are currently lacking.

Apart from the effects of exercise on skeletal muscle, one might also expect exercise to impact tumor growth (Pedersen et al. 2015). In the current study, exercise training did not alter tumor mass,

which confirms previous literature(Ballarò et al. 2019b; das Neves et al. 2016; Khamoui et al. 2016; Ranjbar et al. 2019). In contrast, a recent study showed voluntary wheel running to inhibit tumor growth by increasing the infiltration of natural killer cells into the tumor microenvironment(Pedersen et al. 2016). The potential of exercise to suppress tumor growth is a matter of consistent debate. In this regard, the timing of the exercise intervention relative to tumor induction is probably crucial(Pedersen et al. 2015). Previous studies have provided evidence suggesting that exercise training initiated before, but not after cancer induction, may reduce tumor growth(Pedersen et al. 2016; MacNeil and Hoffman-Goetz 1993). However, this represents a completely different context, i.e. the role of exercise in cancer prevention, rather than in cancer (cachexia) treatment. Consistent exercise prior to cancer onset may promote endogenous resistance to tumor growth by lowering sex and metabolic hormones levels, by suppressing systemic inflammation, as well as via stimulation of immune function(McTiernan 2008). Furthermore, these mechanisms have also been implicated in the association between physical activity and reduced cancer risk(McTiernan 2008). Taken together, our previous observations(Hiroux et al. 2016) and literature data(Pedersen et al. 2016;MacNeil and Hoffman-Goetz 1993) indicate that exercise under specific experimental conditions could inhibit tumor growth. However, the exercise strategy needed to elicit such effect remains unclear. It is also important to note that the C26 model is a fast and aggressive cancer model, which may result in too short time window for exercise to generate physiological adaptations to suppress cachectic degeneration.

In conclusion, C26 cancer cells induced a cachexic phenotype in mice, which is accompanied by a fast-to-slow shift in muscle fiber type composition and by an increased vascularization. Furthermore, C26-induced cancer cachexia blunted the responsiveness of SOL to exercise training. Generally, exercise did not reverse cancer cachexia-induced muscle degeneration nor tumor mass accretion. On the other hand, the voluntary exercise intervention did not exacerbate muscular degeneration either, which indicates that moderate exercise is not necessarily contra-indicated, but there is a need for strategies that improve the muscle's responsiveness towards exercise training, e.g. an anti-inflammatory approach.

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TABLES

Table 1 Effect of cancer cachexia and exercise on tissue mass

	CONs	CON _{EX}	C26s	C26 _{EX}
Total body fat	911 ± 71	702 ± 40 [#]	382 ± 32 [*]	323 ± 24 ^{*,#}
Visceral fat	499 ± 30	411 ± 20 [#]	311 ± 19 [*]	263 ± 24 ^{*,#}
Epididymal fat	318 ± 38	210 ± 21 [#]	39 ± 12 [*]	27 ± 2 ^{*,#}
Brown fat	94 ± 5	81 ± 3	32 ± 6 [*]	33 ± 4 [*]
Spleen	97 ± 3	93 ± 6	240 ± 31 [*]	239 ± 34 [*]
Liver	1184 ± 99	1206 ± 95	1158 ± 107	1180 ± 92
Heart	124 ± 3	128 ± 5	111 ± 3 [*]	107 ± 5 [*]

Data are mean ± SEM and expressed in mg (n=8-9/experimental group). Mice were injected with PBS (CON) or with C26 colon carcinoma cells to induce cancer cachexia (C26). Mice had access to a running wheel in their home cage to stimulate daily exercise training (CON_{EX} and C26_{EX}) or were sedentary (CON_s and C26_s). Mice were sacrificed 18 days after tumor cell injection and different tissues were sampled.

* p < 0.05 CON vs. C26; # p < 0.05 sedentary vs. EX.

Table 2 Effect of cancer cachexia and exercise on grip strength

	CON _s	CON _{EX}	C26 _s	C26 _{EX}
Forelimbs				
Absolute strength (g)				
Pretest	154 ± 7	147 ± 6	156 ± 7	163 ± 8
Posttest	182 ± 4 §	171 ± 2 §	120 ± 9 *,§	119 ± 5 *,§
Relative strength (g·g ⁻¹ BW)				
Pretest	6.3 ± 0.3	6.1 ± 0.3	6.4 ± 0.3	6.7 ± 0.2
Posttest	7.1 ± 0.2 §	7.0 ± 0.2 §	5.9 ± 0.4 *,§	6.1 ± 0.2 *,§
All limbs				
Absolute strength (g)				
Pretest	228 ± 9	224 ± 7	235 ± 9	235 ± 10
Posttest	243 ± 9	241 ± 7	194 ± 16 *,§	183 ± 9 *,§
Relative strength (g·g ⁻¹ BW)				
Pretest	9.4 ± 0.5	9.2 ± 0.2	9.6 ± 0.5	9.7 ± 0.5
Posttest	9.5 ± 0.4	9.8 ± 0.3	9.6 ± 0.6	9.4 ± 0.5

Data are mean ± SEM (n=8-9/experimental group). Mice were injected with PBS (CON) or with C26 colon carcinoma cells to induce cancer cachexia (C26). Mice had access to a running wheel in their home cage to stimulate daily exercise training (CON_{EX} and C26_{EX}) or were sedentary (CON_s and C26_s). Mice were sacrificed 18 days after tumor cell injection. Grip strength of the forelimbs and all limbs was performed before tumor injection, i.e. pretest and at sacrifice day, i.e. posttest.

* p < 0.05 CON vs. C26 at indicated time point; § p < 0.05 Pretest vs. Posttest for CON and/or C26.

Table 3 Effect of cancer cachaxia and exercise on muscle fiber type composition

	CON _S	CON _{EX}	C26 _S	C26 _{EX}
M. gastrocnemius				
Fiber type composition (% of muscle CSA)				
Type IIa	7.2 ± 1.9	8.3 ± 2.2	9.7 ± 1.7	8.9 ± 2.5
Type IIx	7.4 ± 1.0	5.6 ± 1.3	11.2 ± 2.2 *	9.3 ± 1.9 *
Type IIb	84.5 ± 2.9	85.5 ± 3.5	77.7 ± 3.5	80.9 ± 4.5
Fiber type composition (relative fiber number, %)				
Type IIa	12.4 ± 3.0	13.6 ± 3.3	13.2 ± 2.1	13.2 ± 3.0
Type IIx	10.1 ± 0.8	8.2 ± 1.5	14.8 ± 2.2 *	13.0 ± 1.8 *
Type IIb	76.5 ± 3.7	77.4 ± 4.4	70.6 ± 3.6	72.9 ± 4.9
Fiber CSA (μm ²)				
Type IIa	1120 ± 64	1255 ± 94	945 ± 68 *	958 ± 86 *
Type IIx	1482 ± 130	1487 ± 175	944 ± 56 *	1024 ± 67 *
Type IIb	2267 ± 87	2532 ± 205	1484 ± 112 *	1251 ± 64 *
M. quadriceps				
Fiber type composition (% of muscle CSA)				
Type IIa	5.7 ± 1.4	11.0 ± 2.8 #	2.9 ± 0.9	7.0 ± 2.2 #
Type IIx	7.8 ± 1.4	11.1 ± 1.5	9.4 ± 1.7	12.5 ± 2.5
Type IIb	86.5 ± 2.6	77.9 ± 3.7 #	87.7 ± 2.3	80.4 ± 3.8 #
Fiber type composition (relative fiber number, %)				
Type IIa	11.0 ± 2.6	18.0 ± 3.7 #	6.3 ± 1.8	12.5 ± 3.7 #
Type IIx	14.8 ± 2.0	19.0 ± 2.0	18.1 ± 2.1	19.2 ± 2.8
Type IIb	74.2 ± 4.2	63.0 ± 4.4 #	75.6 ± 3.5	68.1 ± 4.8 #
Fiber CSA (μm ²)				
Type IIa	1639 ± 157	1607 ± 94	889 ± 64 *	1087 ± 81 *
Type IIx	1605 ± 84	1658 ± 93	1135 ± 112 *	1294 ± 108 *
Type IIb	3813 ± 214	3597 ± 81	2753 ± 304 *	2581 ± 259 *

Data are mean ± SEM (n=8-9/experimental group). Mice were injected with PBS (CON) or with C26 colon carcinoma cells to induce cancer cachexia (C26). Mice had access to a running wheel in their home cage to stimulate daily exercise training (CON_{EX} and C26_{EX}) or were sedentary (CON_S and C26_S). Mice were sacrificed 18 days after tumor cell injection. Fiber type composition and cross-sectional area (CSA) were determined in m. gastrocnemius and m. quadriceps. Since type I fibers represented less than 1.5% and 0.5% of the muscle cross-sectional area in m. gastrocnemius and m. quadriceps, respectively, data are not shown.

* p < 0.05 CON vs. C26; # p < 0.05 sedentary vs. EX.

FIGURE TITLES AND CAPTIONS

Figure 1 Schematic overview of the experimental design

BW, body weight; SPA, spontaneous physical activity.

Figure 2 C26 mice perform less wheel running activity

Data are mean $\pm$ SEM (n=8-9/experimental group). Mice were injected with PBS (CON) or with C26 colon carcinoma cells to induce cancer cachexia (C26) and had access to a running wheel in their home cage to stimulate daily exercise training (CON_{EX} and C26_{EX}). Mice were sacrificed 18 days after tumor cell injection. Running distance was registered daily. * $p < 0.05$ CON_{EX} vs. C26_{EX} at indicated time points.

Figure 3 Cachexia induces severe body weight loss which is not prevented by voluntary wheel running

Data are mean $\pm$ SEM (n=8-9/experimental group). Mice were injected with PBS (CON) or with C26 colon carcinoma cells to induce cancer cachexia (C26). Mice had access to a running wheel in their home cage to stimulate daily exercise training (CON_{EX} and C26_{EX}) or were sedentary (CON_S and C26_S). Mice were sacrificed 18 days after tumor cell injection. Body weight was measured at various time points. * $p < 0.05$ CON vs. C26 at indicated time points.

Figure 4 Cachexia induces severe muscle atrophy which is not attenuated by voluntary wheel running

Data are mean $\pm$ SEM (n=8-9/experimental group). Mice were injected with PBS (CON) or with C26 colon carcinoma cells to induce cancer cachexia (C26). Mice had access to a running wheel in their home cage to stimulate daily exercise training (CON_{EX} and C26_{EX}) or were sedentary (CON_S and C26_S). Mice were sacrificed 18 days after tumor cell injection. Muscle mass of m. gastrocnemius (GA), m. quadriceps (QU), m. extensor digitorum longus (EDL) and m. soleus (SOL) was determined. * $p < 0.05$.

Figure 5 Effect of cancer cachexia and exercise on *ex vivo* contractile properties of EDL and SOL

Data are mean $\pm$ SEM (n=7-9/experimental group) for specific twitch and tetanus tension of EDL (A and B, respectively) and SOL (C and D, respectively). Mice were injected with PBS (CON) or with C26 colon carcinoma cells to induce cancer cachexia (C26). Mice had access to a running wheel in their home cage to stimulate daily exercise training (CON_{EX} and C26_{EX}) or were sedentary (CON_S and C26_S). Mice were sacrificed 18 days after tumor cell injection. * $p < 0.05$.

Figure 6 Cachexia increases capillary density in GA and QU

Data are mean $\pm$ SEM (n=8-9/experimental group) for capillary density of m. gastrocnemius (A) and m. quadriceps (B). Capillaries are visualized by staining for isolectin conjugated with Alexa Fluor 568 (red). A representative image of each condition of m. gastrocnemius is presented in panel C. The capillary density is determined by the number of isolectin-positive numbers for a given surface. Mice were injected with PBS (CON) or with C26 colon carcinoma cells to induce cancer cachexia (C26). Mice had access to a running wheel in their home cage to stimulate daily exercise training (CON_{EX} and C26_{EX}) or were sedentary (CON_S and C26_S). Mice were sacrificed 18 days after tumor cell injection. Scale bar = 100 μ m. * $p < 0.05$.