



Article

Moderate Aerobic Training Causes Muscle Wasting in a DMBA-Induced Sarcoma Rat Model

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Abstract

Cancer cachexia, characterized by severe body weight loss, negatively affects patient quality of life and survival. Although moderate exercise benefits healthy and chronically ill individuals, and the effect of exercise in cachexia generally appears beneficial, conflicting results have been reported in cancer-associated cachexia. This study examined the effects of moderate aerobic exercise in a rat cancer model, focusing on molecular crosstalk among tumors, serum, and skeletal muscle. Male Sprague-Dawley rats were divided into Non-Cancer, Cancer, and Cancer + Exercise (Ex) groups. Cancer was induced with an intraperitoneal injection of 7,12-dimethylbenz[a]anthracene (DMBA), and the Cancer + Ex group completed an eight-week treadmill regimen. Tibialis anterior muscle, serum, and tumor tissues were analyzed by RNA sequencing. DMBA injection produced sarcoma-like tumors, reduced body weight, elevated inflammatory mediators, and activated muscle atrophy genes (*Fbxo32*). Exercise led to progressive intolerance, further weight loss, lower muscle mass, and larger tumors. Transcriptomic profiling revealed exacerbated cachexia signatures and suppressed energy metabolism genes in exercised cancer rats. Bioinformatic analysis of serum proteins and tissue transcriptomes identified enhanced chemokine-receptor signaling, including pro-tumorigenic (CXCL6_CXCR2) and pro-cachexia (CCL19_CXCR3, CCL5_CCR3, CXCL11_CXCR3) interactions. These findings suggest that in a pro-inflammatory cancer context, late-onset moderate exercise may worsen



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cachexia and stimulate tumor progression. Thus, exercise protocols should be cautiously tailored in cancer settings.

Keywords: cancer cachexia; aerobic exercise; tumor; muscle wasting; transcriptomics

1. Introduction

Cancer cachexia is a complex, multifactorial metabolic syndrome produced by a tumor that leads to weight loss through systemic loss of muscle and body fat, and inflammation [1]. Cancer cachexia is a type of energy balance disorder in which energy intake is decreased, and/or energy expenditure is increased. In general, it is estimated that 50% of cancer patients develop cachexia, and 30% die from cachexia consequences [2]. Weight loss and wasting result from multi-organ crosstalk in cancer, leading to reduced function, reduced response to anti-cancer therapy, and increased mortality [3].

7,2-Dimethylbenz(a)anthracene (DMBA) belongs to the family of Polycyclic Aromatic Hydrocarbons (PAHs) and is a potent carcinogen used to induce several types of cancer. Its carcinogenic effect is due to its ability to break microtubules and degrade DNA (deoxyribonucleic acid) [4]. When applied intravenously, DMBA induces tumors in the thoracic and abdominal region, developing tumors such as fibroadenomas, fibromas, and epithelial cells [5]. Previously, a single intraperitoneal injection of DMBA caused peritoneal sarcoma in Sprague-Dawley rats [6], but there is no investigation into the influence of DMBA on inducing cachexia. DMBA acts through the AhR (Aryl hydrocarbon receptor) receptor, causing cancer-inducing oxidative stress [7]. Cancer cachexia models use various types of induction, such as the application of tumor cells, genetic modifications, and chemically induced cancer models, but still, no standard model has been claimed. In this study, we used the carcinogen DMBA to investigate the potential effects of inducing cachexia.

Various physical exercise protocols have been tested in pre-clinical models and patients with cancer cachexia and have been shown to have beneficial effects, including increasing protein synthesis, mitochondrial content, and decreasing cellular autophagy [8]. Moderate aerobic physical exercise (from 50 to 70% HR max) is well-described to produce positive physiological and muscular adaptations in health and cancer cachexia, decreasing tumor mass, improving muscle mass, and improving survival [9]. Clinical exercise protocols have been shown to reduce inflammation and muscle catabolism through various cellular pathways [10]. A uniform physical exercise protocol for treating patients with cancer cachexia has not yet been established, most likely due to tumor variability, different incidences of cachexia, and the variety of muscles and models studied [10]. Thus, the effect of exercise on cachexia remains unclear.

This study aimed to investigate the effects of a moderate exercise protocol in animals with cancer cachexia and identify potential crosstalk between the tumor and muscle. We hypothesized that moderate aerobic exercise could reduce tumor-induced muscle atrophy, inflammation, and mitochondrial dysfunction and restrain tumor growth. However, our results reveal increased tumor growth and enhanced cachexia with further loss of muscle oxidative capacity, coincident with increased autocrine and paracrine chemokine signaling.

2. Results

2.1. DMBA-Cancer Model Does Not Present a Complete Cachexia Phenotype, Which Was Reached by Aerobic Exercise

Rats were acclimated to housing conditions, then tumors were induced with DMBA (Cancer groups), and a subset was subjected to a moderate-intensity exercise protocol

for eight weeks (Cancer + Ex group) (Figure 1a). Initial body weights were not different between the Non-Cancer and Cancer groups at week 13 (Figure 1b). Rats grew throughout the study (Figure 1b); however, body weights at euthanasia demonstrated lower tumor-free body weight in the Cancer group versus Non-Cancer ($p = 0.038$), with body weight further reduced in the Cancer + Ex group compared to Cancer ($p = 0.006$) and Non-Cancer ($p < 0.0001$, Figure 1b). Aerobic exercise exacerbated tumor growth ($p = 0.0177$) (Figure 1c). Food intake remained unchanged over time (Figure 1d). The Cancer group presented higher pancreas and kidney weights compared to both Non-Cancer and Cancer + Ex groups, with no change in liver weight (Figure 1e).

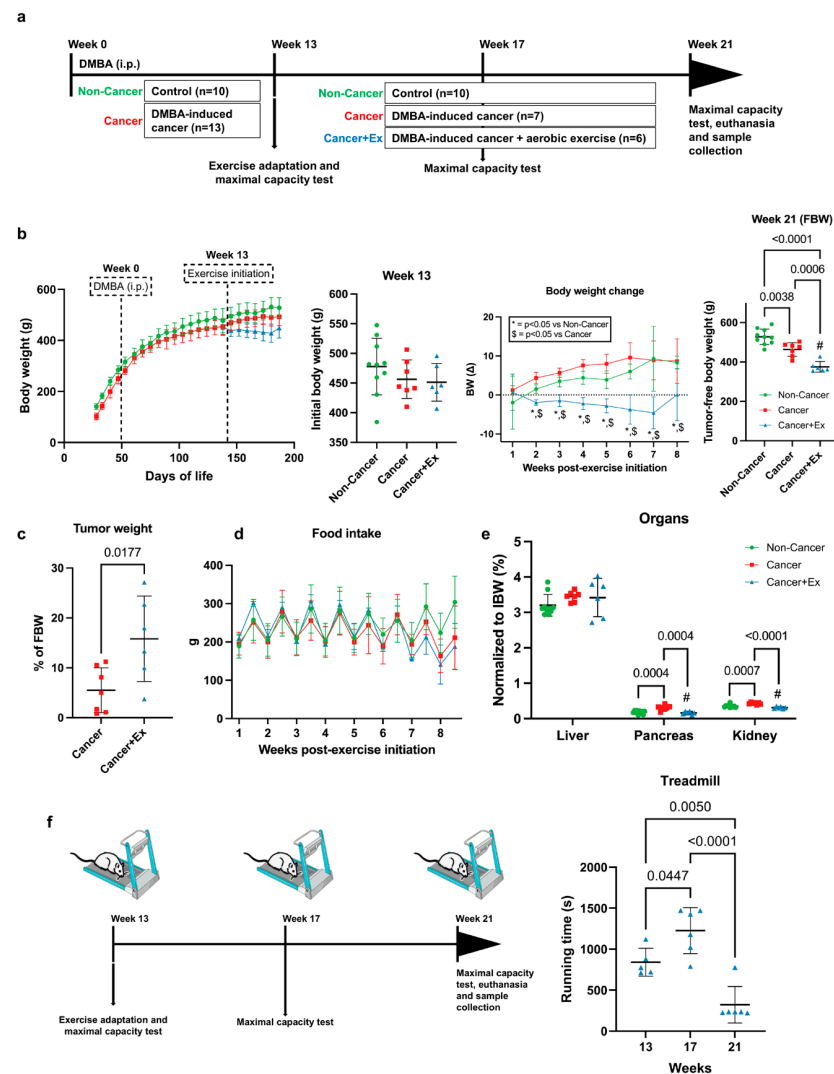


Figure 1. Aerobic exercise exacerbated body weight loss, increased tumor weight, and presented exercise intolerance. (a) Experimental design. (b) Body weight over time, initial body weight, body weight change, and tumor-free body weight at euthanasia, where # = Cancer + Ex vs. Cancer group using unpaired t -test ($p = 0.0004$). (c) tumor weight as a fraction of total body weight, analyzed using an unpaired t -test. (d) food intake. (e) Liver, Pancreas, and Kidney weights normalized by IBW. Non-Cancer ($n = 10$); Cancer ($n = 7$); and Cancer + Ex ($n = 6$). (f) Visualization of aerobic exercise maximal capacity assessments and decreasing exercise tolerance in the Cancer + Ex group over time. Week 13 ($n = 5$), week 17 ($n = 6$), and week 21 ($n = 6$). # = Significant difference between the week 17 and 21 using unpaired t -test, $p = 0.0001$.

During the eight weeks of exercise intervention, we verified the time of exercise tolerance in three assessments over time, in the first, fourth, and eighth week of exercise

(Figure 1e), and observed a decline in exercise tolerance time in Cancer + Ex on the last assessment (321.67 ± 223.13 s) compared to the second (1227.00 ± 280.94 s, $p < 0.0001$) and first assessment (841.40 ± 170.26 s, $p = 0.0050$), suggesting development of severe fatigue (Figure 1e). Loss of exercise tolerance was reflected in the dysregulation of genes involved in the glycolysis pathway (Figure S1a) and oxidative phosphorylation genes (Figure S1b). Significant loss of these genes was nearly exclusive to Cancer + Ex, suggesting that aerobic exercise downregulated genes responsible for energy generation by mitochondria and depleted energy resources. These results suggest that aerobic exercise in advanced cancer is no longer tolerated and results in exercise intolerance.

Extensor Digitorum Longus (EDL) and soleus weights were increased in the Cancer group compared to the Non-Cancer and the Cancer + Ex group ($p < 0.0001$ for all comparisons) (Figure 2a). Tibialis anterior and gastrocnemius weights were decreased in the Cancer + Ex group compared to Non-Cancer ($p = 0.0005$ for tibialis anterior, $p < 0.0001$ for gastrocnemius) and Cancer ($p = 0.0001$ for tibialis anterior, $p = 0.0003$ for gastrocnemius) groups (Figure 2a). The raw data is also provided in Table S1. The Cancer group did not present evidence of atrophy or fibrosis when compared to the Non-Cancer group, suggesting that this model was not enough to induce cancer cachexia (Figure 2c). Interestingly, the Cancer + Ex group presented more collagen deposition when compared to the Non-Cancer group ($p = 0.061$). Using frozen section staining for NADH-TR (Figure S1c), we observed a reduction in CSA in the Cancer + Ex group compared to the Non-Cancer group ($p = 0.0035$) and Cancer group ($p = 0.0103$) (Figure 2c).

The skeletal muscle ubiquitin ligase *Fbxo32*, the IL-6 signaling pathway member *Il6st*, and the growth hormone *Igf1* were upregulated in the Cancer and Cancer + Ex groups compared to Non-Cancer (Figure 2d), while *Casp3*, *Igf1r*, *Map3k14*, *Pdpk1*, and *Tnfrsf1a* were upregulated only in the Cancer + Ex group. Furthermore, genes involved in autophagy (Figure 2d) and oxidative stress (Figure 2d) were also upregulated or downregulated in the Cancer and Cancer + Ex groups compared to the Non-Cancer group, but mostly in the Cancer + Ex group. Tibialis anterior muscle weights were reduced proportionately to tumor mass in Cancer (Pearson $r = -0.7180$, $p = 0.0692$) and significantly in Cancer + Ex ($r = -0.9063$, $p = 0.0156$) (Figure 2e). Muscle mass reduction was also correlated to reduced kidney and adipose tissue mass (Figure 1f). This data indicates that the DMBA rat cancer model did not produce a pre-cachexia condition, and it was aggravated by exercise.

Finally, no differences were found in oxidative damage among the groups, except for more SOD activity in the Cancer + Ex group (Figure S1d). Taken together, those results suggest that the DMBA-cancer model presented body weight loss (tumor-free body weight) and adipose tissue loss but did not present muscle loss. On the other hand, moderate aerobic exercise exacerbated body weight loss, muscle loss, adipose loss, and increased tumor weight.

2.2. The Muscle Cachexia Response Was Exacerbated by Aerobic Exercise

Expression profiling of the tibialis anterior identified 18,939 transcripts across all groups. An unsupervised principal component analysis of all transcripts revealed some heterogeneity within groups but overall separation among groups (Figure 3a). Filtering for differentially expressed genes with fold change $> |2|$ and adjusted p -value < 0.05 revealed 523 upregulated and 30 downregulated genes between Cancer and Non-Cancer (Figure 3b). The Cancer + Ex vs. Non-Cancer contrast showed 1276 upregulated and 744 downregulated genes (Figure 3c). Despite the significant difference in muscle mass, the contrasting Cancer + Ex group vs. Cancer directly presented only 10 upregulated and 16 downregulated genes due to sample heterogeneity (Table S2).

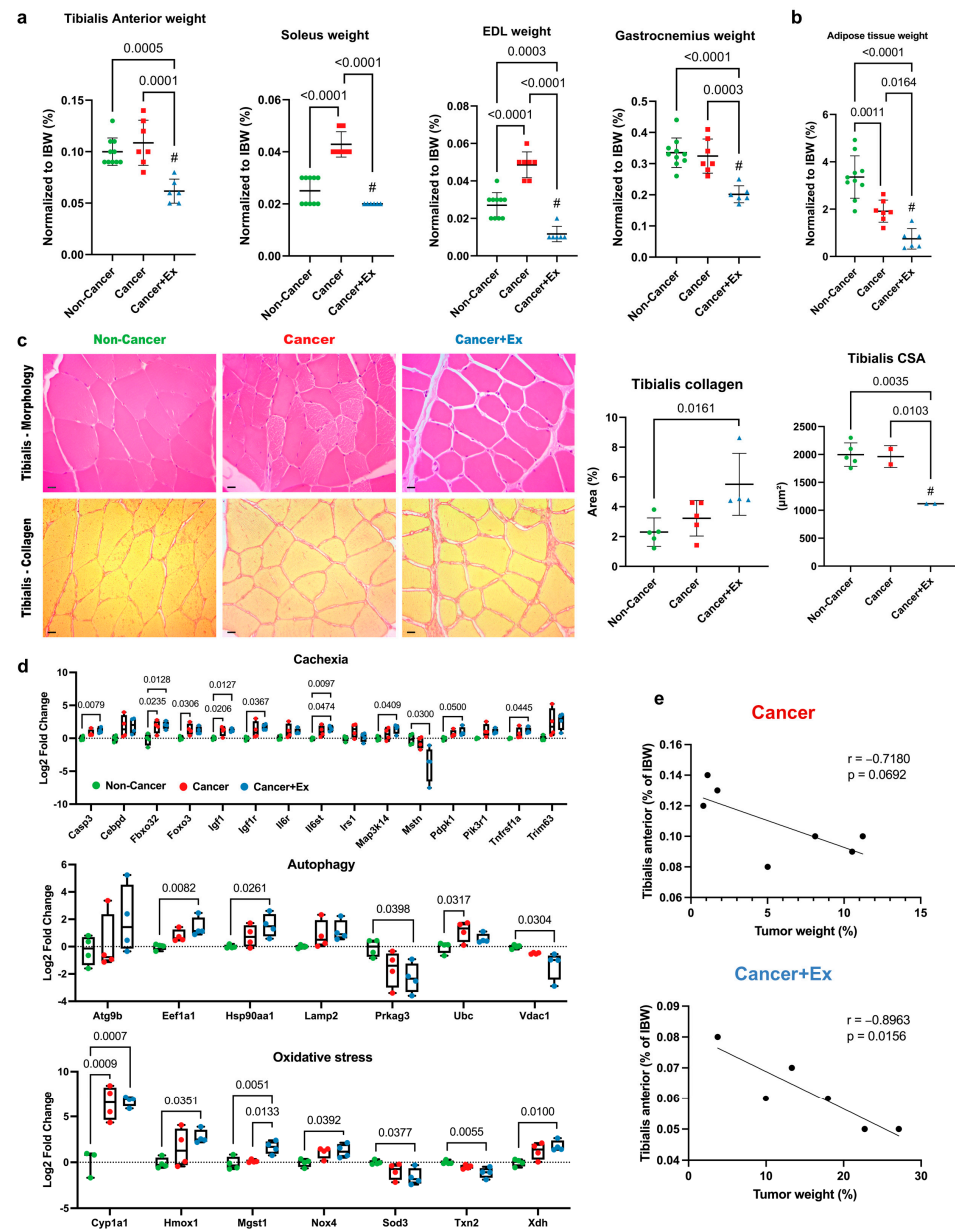


Figure 2. Aerobic exercise causes muscle weight loss and increased muscle collagen deposition, causing the exacerbation of cachexia phenotype, which was confirmed by the cachexia genes. (a) skeletal muscle weight normalized to IBW. Tibialis anterior ($\# = \text{Cancer} + \text{Ex}$ vs. Cancer group unpaired t -test, $p = 0.0007$); soleus ($\# = \text{Cancer} + \text{Ex}$ vs. Cancer group unpaired t -test, $p = <0.0001$); EDL ($\# = \text{Cancer} + \text{Ex}$ vs. Cancer group unpaired t -test, $p = <0.0001$); gastrocnemius ($\# = \text{Cancer} + \text{Ex}$ vs. Cancer group unpaired t -test, $p = 0.0004$). (b) Adipose tissue weight. $\# = \text{Cancer} + \text{Ex}$ vs. Cancer group unpaired t -test, $p = 0.0007$. (c) Tibialis anterior histology. $\# =$ Significant difference between the Cancer + Ex and Cancer groups using unpaired t -test, $p = 0.0260$. In histology analysis, the number of animals per group is lesser given priority over other techniques. Collagen assay = Non-Cancer ($n = 5$), Cancer ($n = 5$), and Cancer + Ex ($n = 4$). Hematoxylin and Eosin images are only representative, Bar: $20\ \mu\text{m}$, resolution of $400\times$. CSA analysis = Non-Cancer ($n = 5$), Cancer ($n = 2$), and Cancer + Ex ($n = 2$). (d) Cancer cachexia-related genes according to the Skeletal Muscle Wasting in Cancer Cachexia dataset provided by the Elsevier_Pathway_Collection library. Autophagy-related genes according to the Reactome_2022 library. Oxidative stress-related genes according to the BioPlanet_2019 library. All the plotted genes are significantly different from the Non-Cancer compared to Cancer and/or Cancer + Ex (All DEGs can be found in Table S2). Non-Cancer ($n = 4$); Cancer ($n = 4$); Cancer + Ex ($n = 4$). (e) Person simple linear regression of muscle wasting and tumor mass in the Cancer group ($n = 7$), and Cancer + Ex group ($n = 6$).

To further interrogate similarities and differences, unsupervised clustering of all differentially expressed genes and samples revealed two clusters with 1405 (first cluster) and 752 genes (second cluster) (Figure 3d—top and bottom). Pathway enrichment analysis of the first indicated upregulation of immune, innate immune, platelet activation, and cytokine response, including Interleukin-6, all of which tended to increase from Non-Cancer, Cancer, to Cancer + Ex samples (Figure 3e). The second cluster revealed downregulation of energy-producing pathways, including the citric acid (TCA) cycle, respiratory electron transport, and oxidative phosphorylation, which tended to decrease from Non-Cancer, Cancer, to Cancer + Ex (Figure 3f). Meta-analysis of Cancer + Ex vs. Non-Cancer and Cancer vs. Non-Cancer revealed an overlap of 395 genes (Figure 3, Table S3). Pathway enrichment analysis of the overlap revealed the upregulation of hallmark pathways related to inflammation, wound healing, and innate immune response as the most common pathways between the comparisons, while energy-producing pathways such as mitochondrial inner membrane, mitochondrial respiratory chain, generation of precursor metabolites, and energy were disproportionately downregulated in the Cancer + Ex group (Table S3). A few unique pathways were identified among genes upregulated exclusively in Cancer + Ex; rather, the same pathways were enhanced over Cancer alone.

The previous analysis showed participation of immune and inflammatory-related pathways in the transcriptome of skeletal muscle. To further address this observation, we performed immune cell deconvolution using the CIBERSORTx web tool to evaluate the distribution of 22 immune cells based on our tibialis gene counts. Confirming what we saw previously on Figure 3 and Table S3, the Cancer group presented a higher presence of immune cells compared to the Cancer + Ex group, such as mast cells resting (Figure S2a). The Cancer and Cancer + Ex group presented a reduction in plasma cells compared to the Non-Cancer group, while only the Cancer + Ex group presented a reduction in dendritic cells resting compared to the Non-Cancer group (Figure S2a). Regarding the Macrophage M2 population, the Cancer group presented more expression when compared to the Non-Cancer group (Figure S2a), corroborating that the Cancer + Ex group might have an inflammatory microenvironment. The tumor immune cell deconvolution did not exhibit any significant difference between the Cancer and Cancer + Ex group (Figure S2b).

2.3. The Tumor Transcriptome Reveals the Identification as a Rhabdomyosarcoma and a Small Amount of Gene Alteration by Aerobic Exercise

All DMBA-treated rats included in this analysis had visible, palpable tumors at the injection location (intraperitoneal cavity/wall) by week 13. Some rats also developed tumors at distant sites, such as the head and chest. This was not different between the no-exercise and exercise groups. Histology revealed tumors with pronounced extracellular matrix and spindled cells with vesicular nuclei and occasional mitoses (Figure 4a, higher magnification shown in Figure S3a). Although tumors in the Cancer + Ex group were larger, no differences were notable by histology. A total of 19,146 transcripts were identified in tumors. Enrichment analysis of the top 1000 genes by mean expression revealed that myoblasts and myofibroblasts' signatures were the most over-represented (Figure 4b). Furthermore, we confirmed the presence of rhabdomyosarcoma markers [11] (Figure S3b). This data suggests that the DMBA-induced rhabdomyosarcoma-like tumors.

Aerobic exercise resulted in a few differentially expressed genes, with 132 downregulated and 24 upregulated genes compared to the Cancer group (Figure 4d, Table S4). We performed an enrichment analysis to explore the two distinct clusters identified in the heatmap (Figure 4d). Physical exercise increased genes in the ERBB2-EGFR signaling pathway (driven in part by a 10.6-fold increase in *Tgfa*, $p = 0.0002$), collagen biosynthesis, modifying enzymes (Figure 4e), and reduced genes involved in chemokine/chemokine receptor (including reduced *Ccl19*, *Ccl11*, *Ccl21*, and *Cxcl14*) and regulation of the ERK1

and ERK2 cascade pathways (Figure 4f). To further investigate the influence of exercise intolerance on the tumor environment, we performed an antibody assay and verified the most altered proteins (FC > 2 threshold) in the tumor of the Cancer + Ex group compared to the Cancer group (Figure S3c,d). Although we found a downregulation of the chemokine pathway in the tumor transcriptome, some chemokines were more abundant in the Cancer + Ex, such as *Ccl3*, *Cxcl2*, and *Lix*. Corroborating the upregulation of genes involved in collagen biosynthesis, modifying enzymes, and extracellular matrix organization, biological processes, we found a greater abundance of proteins that may be involved in the regulation of extracellular matrix organization, including *Vegf*, *Mmp3*, and *Mmp9*. Among differentially present tumor proteins, only Pref-1/DLK1/FA1 was differentially expressed in the tumor transcriptome (Figure S3e).

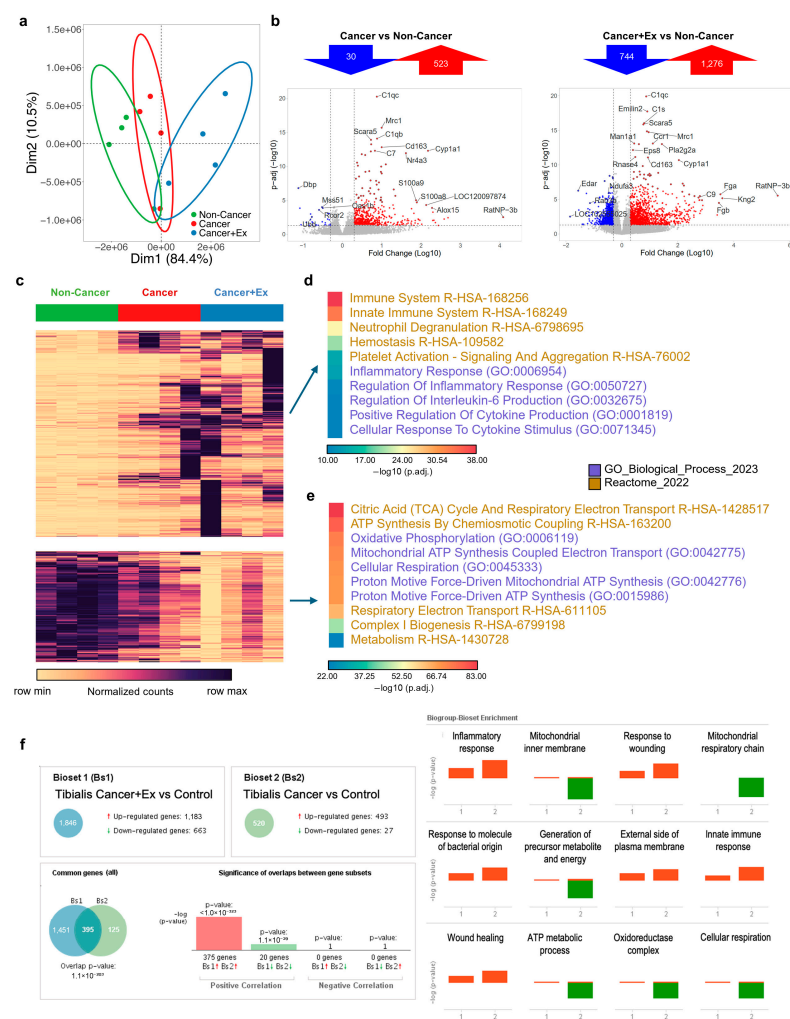


Figure 3. The tibialis transcriptome revealed activation of inflammation, inhibition of oxidative metabolism, and induction of known cachexia markers. (a) Muscle transcriptome principal component analysis. Each dot represents an individual sample. (b) Volcano plots of tibialis gene expression in Cancer vs. Non-Cancer, and Cancer + Ex vs. Non-Cancer comparison. A red arrow pointing upwards represents upregulated genes. A blue arrow pointing downwards represents downregulated genes. (c) Heatmap of differentially expressed genes with two cluster (top and bottom). (d) Functional enrichment analysis of the top cluster in the heatmap (indicated by the arrow), showing altered pathways ranked by significance. (e) Functional enrichment analysis of the bottom cluster in the heatmap (indicated by the arrow) ranked by significance. (f) Meta-analysis of the transcriptome between Cancer and Cancer + Ex and contrast of Gene Ontology pathway differences. Red: Up-regulated pathways; Green: Down-regulated pathways. Non-Cancer (n = 4); Cancer (n = 4); Cancer + Ex (n = 4).

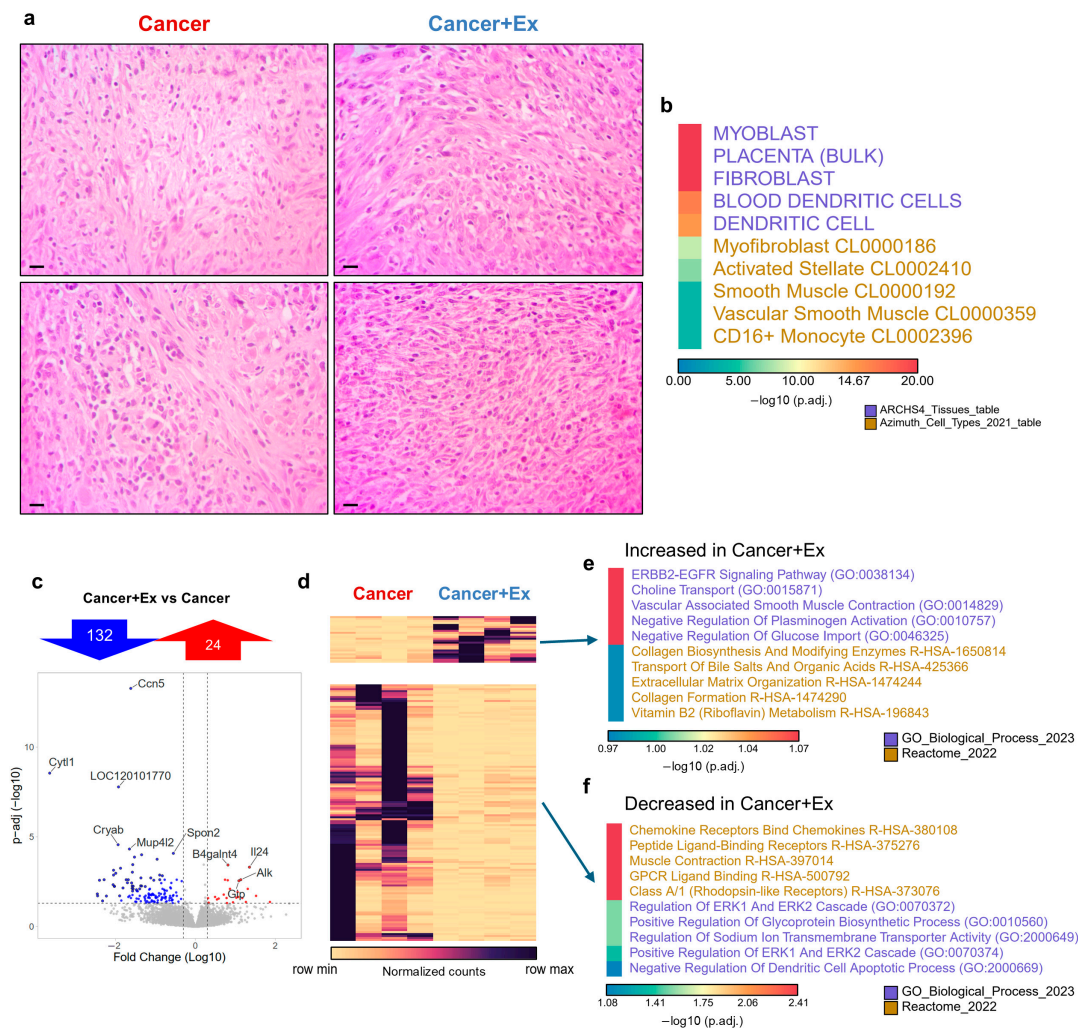


Figure 4. Tumor identity analysis suggests a rhabdomyosarcoma with a transcriptome only modestly affected by exercise. (a) Histology of tumors. Bar = 20 μ m, 40 $\times$ magnification. (b) Tumor top 1000 genes enrichment for cell type. (c) Volcano plot for all genes FC ≥ 1.2 and FDR ≤ 0.05 . A red arrow pointing upwards represents upregulated genes. A blue arrow pointing downwards represents downregulated genes. (d) heatmap for all genes FC ≥ 1.2 and FDR ≤ 0.05 , showing two cluster (top and bottom). (e) Functional enrichment analysis of the top gene cluster of the heatmap (indicated by the arrow). (f) Functional enrichment analysis of the bottom gene cluster of the heatmap (indicated by the arrow). Cancer (n = 4); Cancer + Ex (n = 4).

2.4. Serum Proteins and Potential Crosstalk in the Macroenvironment of Cachexia

Out of 79 analyzed proteins in the serum, 40 were changed more than twofold, and all were increased in Cancer and were similar in the Cancer + Ex group (Figure 5a). Although the ERBB2-EGFR signaling pathway was upregulated, exercise decreased serum EGF (a classical EGFR ligand) compared to the Cancer group (Figure 5a), suggesting the involvement of an additional gene in this critical pathway for tumor growth. Many chemokines were increased, including CCL2, CCL5, CCL11, CCL17, CCL20, CCL21, and LIX (Figure 5a). Among them, the *Ccl2*, *Ccl5*, *Ccl11*, *Ccl17*, *Ccl20*, and *Ccl21* were more expressed in tumors than in tibialis (Figure 5b). Little data support the enhanced expression of these serum proteins from tibialis with Cancer or Cancer + Ex, suggesting the tumors are the source of many differentially present serum proteins. Others, including *Wisp1*, *Nov*, and *Cxcl5*, were expressed neither in the tumor nor the tibialis anterior (Figure 5b), suggesting that cancer elicited expression of these factors from different tissues.

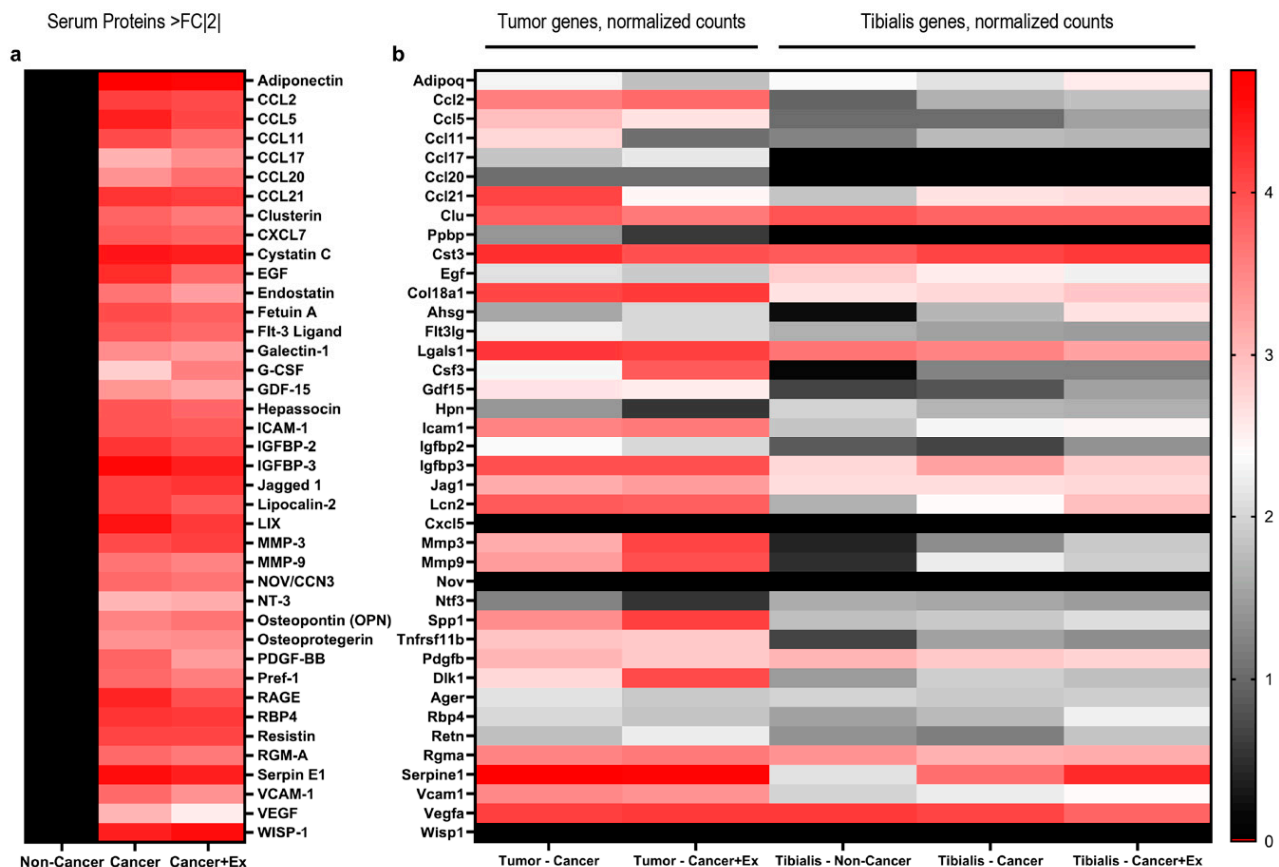


Figure 5. Alterations in serum proteins versus tumor and tibialis transcriptomes. **(a)** Cytokine array of serum proteins with more than 2-fold change. Non-Cancer (n = 1); Cancer (n = 1); Cancer + Ex (n = 1). **(b)** Respective gene expression in the tumor and tibialis. The values were log-transformed for plotting. Non-Cancer (n = 4); Cancer (n = 4); Cancer + Ex (n = 4).

2.5. The Chemokine Pathway Was Activated in Both Tissues, and It Is a Candidate to Mediate Muscle Wasting in Tumor–Muscle Crosstalk

We performed an interaction analysis using differentially expressed genes to find a possible interaction between the tumor secretome and the tibialis anterior muscle. First, we performed an autocrine analysis, looking for ligands and receptors in the same tissue. In the tibialis, five strong interactions were found: Ccl19_Cxcr3, Ccl5_Ccr3, Cxcl11_Cxcr3, F2_F2rl2, and GDNF_Slc44a5; in the tumor we identified Bmp2_Bmpr1b, and Cxcl6_Cxcr2 (Figure 6a). Enrichment analysis of those genes presents the chemokine receptors that bind chemokines as the most involved ontology (Figure 6b). We performed the same analysis using only the FC > 2 filter to query potential paracrine communication. The tumor-to-tibialis presented 12 interactions (Figure 6c). The enrichment analysis of the tibialis receptors showed that they are mainly involved in the Receptor-Mediated Endocytosis biological process and GPCR Ligand Binding pathway (Figure 6d). The tibialis-to-tumor presented 24 interactions (Figure 6e), and tumor receptors were mostly involved in the Extracellular Matrix Organization biological process and integrin-mediated signaling pathway (Figure 6f).

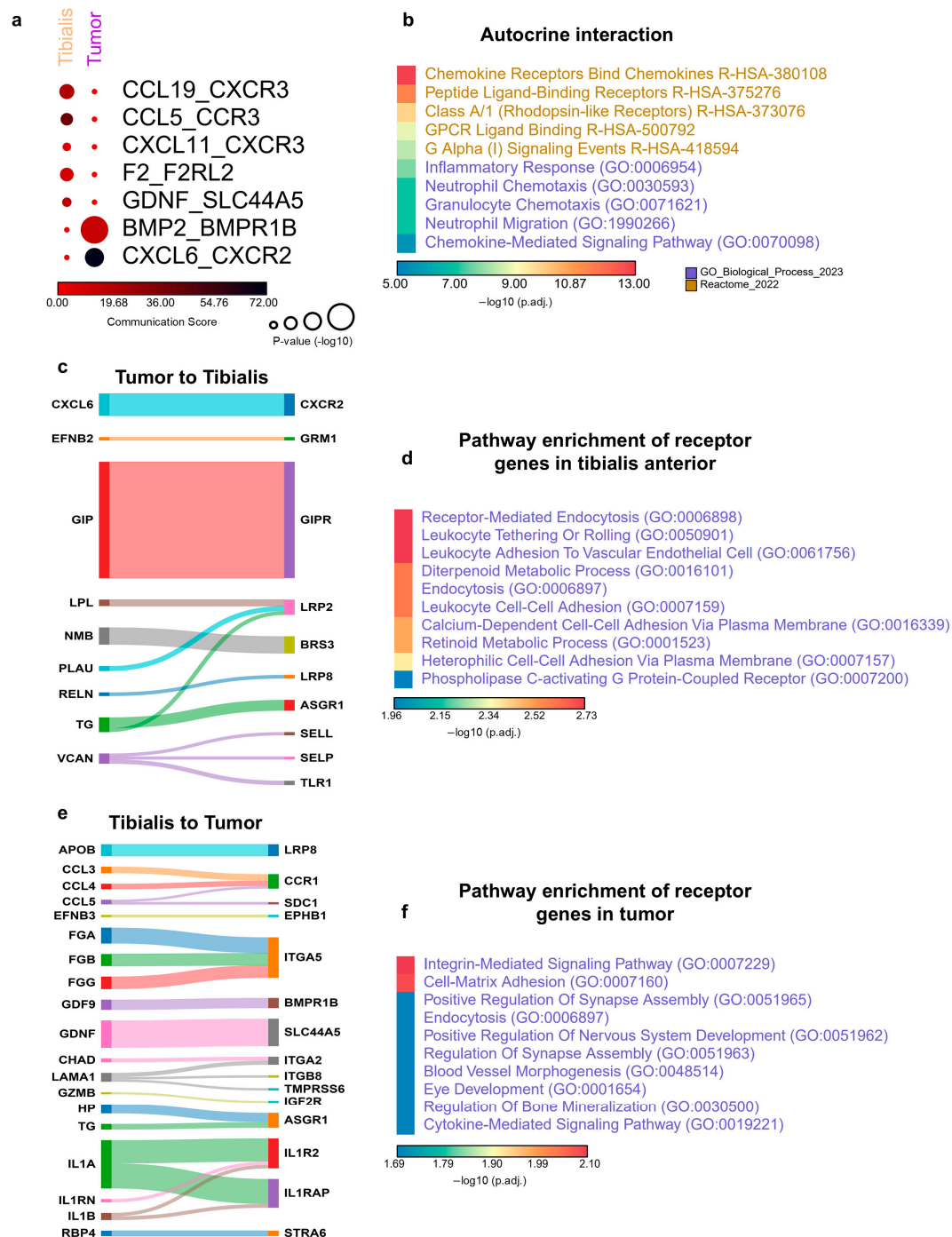


Figure 6. Interactome analysis reveals possible crosstalk within and among the tumor and tibialis. (a) Possibly involved pathways in autocrine communication in the Cancer + Ex vs. Cancer comparison for tumor and tibialis anterior tissues. (b) Functional enrichment analysis of the genes presented in (a). (c) Possible pathways involved in paracrine communication in the Cancer + Ex vs. Cancer comparison from tumor to tibialis. The node thickness represents the communication score. (d) Functional enrichment analysis of the genes presented in c as an Alluvial Plot. The node thickness represents the communication score. (e) Possible involved pathways in paracrine communication in the Cancer + Ex vs. Cancer comparison from the tibialis to the tumor. The node thickness represents the communication score. (f) Functional enrichment analysis of the genes presented in (e) as an Alluvial Plot. The colors are used only to visually distinguish different ligand–receptor interactions and do not carry biological meaning. Cancer (n = 4); Cancer + Ex (n = 4).

3. Discussion

This study aimed to investigate the effect of physical exercise in the treatment of cancer cachexia. In contrast to reports of the beneficial effects of physical exercise on cachexia and muscle phenotypes in models and patients with cancer, our late-onset moderate exercise protocol resulted in reduced exercise tolerance, increased tumor growth, and accelerated muscle wasting. The inflammatory and catabolic pathways present in rats with tumors were enhanced by exercise, while energy production pathways were impaired. Moderate exercise promoted pro-tumorigenic and pro-cachectic chemokine signaling pathways within and between the tumor and muscle. Thus, physical exercise enhanced autocrine and paracrine interactions in the tumor microenvironment, driving tumor growth and muscle wasting (Figure 7).

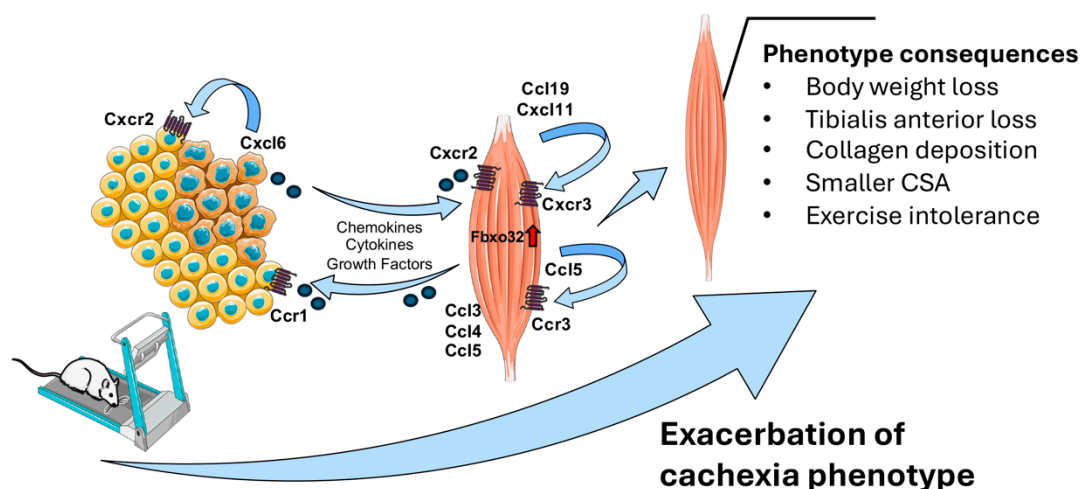


Figure 7. Graphical abstract of the tumor–muscle crosstalk. This figure illustrates the interactions between chemokines, cytokines, and growth factors between the tumor and skeletal muscle. This interaction was efficient in inducing a cachexia phenotype by upregulation of cachexia canonical genes, such as *Fbxo32* (arrow pointing upwards). Aerobic exercise exacerbated the cachexia phenotype, increasing the tumor weight and accelerating muscle wasting. The autocrine communication in the tumor and tibialis was mainly mediated by chemokines. In tumors, the *Cxcl6* and *Cxcr2* receptor cascades were candidates to mediate the effects. In tibialis, *Ccl19* and *Cxcl11* ligands were candidates for binding the *Cxcr3* receptor, as well as the *Ccl5* ligand for binding the *Ccr3* receptor. Figure made using Servier Medical Art (<https://smart.servier.com>), licensed under CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>) (accessed on 3 December 2025)).

The Cancer + Ex group presented a significant reduction in exercise tolerance over time. Although aerobic exercise generally enhances oxidative capacity in muscle, surprisingly, genes involved in oxidative phosphorylation and glycolysis/gluconeogenesis pathways were reduced by exercise. Some critical genes in glycolysis/gluconeogenesis, such as *Pdhb* (that encodes the enzyme pyruvate dehydrogenase), were downregulated, although *Pck1* (phosphoenolpyruvate carboxykinase 1)—the first rate-limiting enzyme in gluconeogenesis was upregulated. *Pck1* upregulation is suggested to be a compensatory mechanism associated with endurance capacity and could provide a catabolic advantage during cachexia [12]. Regardless, the running stimulus led to tissue loss, downregulated mitochondria-related genes, and evidence of heightened inflammation.

Exercise can have different effects on tumor development depending on the exercise variations. We are not the first to report that exercise can increase tumor growth. Using a C26 mouse treated with chemotherapy plus moderate aerobic exercise, Ballarò et al. reported improvement in tibialis anterior weight and reduction in *Fbxo32* and *Trim63* gene expression. Still, these effects were accompanied by more significant tumor growth,

even when initiating the protocol before tumor implantation [13]. Moreover, mortality was accelerated in the exercised group, and the authors suggest that in the late stages of cachexia, exercise is no longer tolerated [13]. A suppressive effect of exercise on tumor growth has been observed in studies that initiate exercise from fourteen to twenty days before tumor induction [14], and this effect was attenuated when physical exercise was initiated at the same time as tumor induction [15]. Positive results were found in female Sprague-Dawley rats exercised prior to nitrosomethylurea (NMU—another chemical carcinogen) intraperitoneal administration, which had a protective effect, reducing the incidence of tumors compared to the sedentary [16]. These results suggest that the time of exercise initiation has a considerable influence on tumor outcome.

Some evidence from the literature may help explain our findings regarding the effects of exercise. Vanderveen et al. [17] used the $Apc^{Min/+}$ model and implemented voluntary exercise at 13 weeks of age, when tumors were already established. Despite the advanced disease stage, the exercised mice showed improved treadmill fatigue time and grip strength. In another study using the same model, exercise performed on a treadmill at moderate intensity initiated changes in inflammatory profiles in non-tumor and tumor-border tissues; however, these effects were observed when exercise started at 3.5 weeks of age, a time point at which tumors are not yet fully established [18]. Importantly, in the $Apc^{Min/+}$ model, mice with mild cachexia typically do not exhibit body weight loss, and those with moderate cachexia show a delayed decline in body mass. This pattern suggests that the Cancer group in our study may eventually progress to weight loss as cachexia evolves. Baltgalvis et al. [19] also evaluated voluntary running starting at 5 weeks of age and reported that only mice classified as having severe cachexia showed an early reduction in wheel-running distance and peak velocity, indicating that the severity of cachexia is closely linked to exercise capacity. In contrast, in our study, only the Cancer + Ex group exhibited more pronounced cachexia symptoms. Together, these comparisons suggest that the timing of exercise initiation during the development of cancer-associated cachexia is a critical factor determining whether exercise mitigates or exacerbates cachexia progression.

Our study followed the recommendations of exercise intensity for cancer patients, using 60% of the intensity of exercise (moderate) [20], but it led to poorer outcomes, including greatly increased tumor growth. The ERBB2-EGFR signaling pathway was upregulated by exercise. *ErbB2* and *Egfr* gene overexpression is correlated with poor clinical outcomes and large tumor size in several cancer types [21]. Ligand binding to EGFR causes a heterodimerization formation with ERBB2, inducing phosphorylation and activation of downstream effectors, such as PI3K/Akt and STAT3 signaling, to promote tumor cell survival and proliferation [22]. The Cancer + Ex group has lower abundance of serum EGF. Accattatto et al. [23] also reported reduced EGF serum levels after physical exercise. However, exercise increased tumor expression of *Tgfa* encoding TGF- α , which acts as a tumor promoter and accelerates mammary neoplasia in previous DMBA-cancer models [24]. TGF- α is also an EGFR ligand; thus, *Tgfa* may promote tumor growth via autocrine signaling [25]. Collagen biosynthesis and formation pathways, and extracellular matrix organization, were also increased by exercise and could contribute to tumor growth.

Taking this into consideration, Pigna et al. [26] initiated exercise treatment on the same day of tumor transplantation in the C26-bearing mice model, and, to avoid exercise-induced muscle damage caused by the forced exercise protocol, mice were allowed to run on individual voluntary wheels in their cages. Results were positive, showing the control of autophagy markers and genes in muscle. Here, we used an imposed, forced exercise protocol applied not during the tumor initiation, but three months later, which accelerated cachexia rather than reducing it. Pereira et al. [27], using a very similar aerobic exercise protocol, found an initial improvement in exercise tolerance in healthy mice, but

overtraining by increasing the volume, decreasing the recovery time between sessions, and applying a grade to the treadmill, caused a decrease in exercise tolerance in the same mice. We also observed an initial positive response to training (but not significant) and a significant decline, indicating progressive exercise intolerance in cancer animals. Our data indicate that the effects observed in this study might be due to overtraining in rats at a late stage of cancer, not a failure of moderate exercise itself.

Previous studies have shown that forced treadmill exercise can raise the serum corticosterone level (a stress hormone) in Sprague-Dawley rats [28]. Also, mice subjected to forced treadmill presented anxious behavior and more fecal corticosterone compared to no exercise [29]. Stress induced by isolation caused higher levels of corticosterone compared to the cancer group without isolation, and it was associated with larger tumor volume in rats [30]. Glucocorticoids can regulate tumor development, promoting proliferation [31]. Even though we did not perform an assay for stress, this evidence suggests that our protocol was stressful, and that may have promoted the tumor growth in the Cancer + Ex group compared to the Cancer group alone.

Our results suggested that exercise increased CXCL6-CXCR2 autocrine communication in tumors and from tumors to muscles. CXCL6-CXCR2 is a known axis in tumor development, regulating the tumor microenvironment and promoting growth, migration, and invasion [32]. The CXCR2 receptor is implicated in skeletal muscle atrophy in pancreatic cancer cachexia models [33] and was upregulated in muscle by exercise in this study. Furthermore, there is evidence that increased CXCR2 mRNA after exercise induces angiogenesis in healthy individuals [34]. Thus, exercise could promote CXCR2 signaling in muscle downstream of CXCL6 expression from tumors to enhance muscle loss.

Taken together, several methodological aspects of our study may explain why our findings differ from reports showing beneficial effects of exercise in other cancer models. First, the DMBA-induced carcinogenesis model presents high tumor heterogeneity, rapid progression, and a pronounced systemic inflammatory environment, which differs substantially from genetic models that develop cachexia more slowly and predictably. Second, exercise was initiated at a relatively late stage of tumor progression, when skeletal muscle was already metabolically compromised and therefore less able to tolerate additional physiological demands. Third, the use of a forced treadmill protocol at 60% of Pmax, although classified as moderate intensity, imposed a fixed workload that may have exceeded the adaptive capacity of cachectic muscle and contributed to increased physiological stress. These combined factors offer a plausible explanation for why, in our study, aerobic exercise did not promote the protective outcomes reported previously in rodent cancer models [35], and in patients following adjuvant chemotherapy [36]. Importantly, these findings do not suggest that exercise is inherently detrimental in cancer or cachexia. Instead, they indicate that exercise benefits are highly context dependent and require careful evaluation of disease stage, tumor characteristics, and the individual's metabolic reserve before defining an intervention. Therefore, rather than disqualifying exercise as a therapeutic strategy, our results reinforce the need for tailored exercise prescriptions that consider the patient's specific clinical condition to optimize safety and expected outcomes.

This study has limitations. Because only male rats were included, the results cannot be fully extrapolated to females, which is relevant given the known sex differences in tumor biology and responses to exercise. A single training study design does not allow for differential effects of exercise timing, dose, or type. Analyses of proteins in serum and tumor were not sufficiently powered to define significance, and gene expression and pathway analyses are suggestive but do not provide definitive evidence of molecular mechanisms. Furthermore, the enhanced muscle loss seen with exercise might be due to increased tumor growth at the expense of muscle recovery due to shunting of nutrition and

energy stores. Also, it is challenging to distinguish the effects of the tumor from the DMBA itself. DMBA causes immunotoxicity over time by affecting the spleen and thymus [37]. The induction of DMBA toxicity can take 8 to 10 weeks, starting to affect the liver, kidney, and brain, and reducing the hemoglobin levels [38]. It is hard to know if it is cancer or the delayed DMBA effects since we have started the exercise after ~90 days, enough time to induce all the effects mentioned above. When exercise started, all the DMBA effects were associated with the tumor effects. Despite these limitations, our data definitively demonstrate a deleterious effect of exercise in this model of cancer and cachexia. This model can also generate metastases in some animals, although this was not quantified. Finally, the absence of a non-cancer aerobic exercise group limits our ability to isolate the independent effects of aerobic training under comparable experimental conditions. Despite these limitations, the overall pattern of our results indicates a deleterious effect of aerobic exercise (60% of Pmax) in this model of DMBA-induced cancer and cachexia.

4. Materials and Methods

4.1. Animals and Experimental Design

Thirty male Sprague-Dawley (SD) rats aged four weeks, weighing on average 130 g, were obtained from the Biotherism Center of the State University of Campinas (CEMIB/UNICAMP). The animals received water and a solid diet ad libitum (Nuvilab, Colombo, Paraná, Brazil). Animals were housed in polypropylene cages with a solid bottom lined with shavings, accommodating 2–3 rats each, and were weighed weekly. They were kept in a room with controlled lighting (12 h of light and 12 h of darkness) and temperature (20 to 25 °C). The animals were divided into three experimental groups: Non-Cancer (group without tumors, $n = 10$); Cancer (DMBA-induced cancer, $n = 10$); and Cancer + Ex (cancer induced by DMBA and subjected to 8 weeks of aerobic physical training, $n = 10$). During the experimental protocol, some of the animals died or did not develop tumors. These animals were removed from all statistical analyses: 3 in the Cancer group and 4 in the Cancer = Ex group. Only animals with tumors that successfully developed in the retroperitoneal area and survived until the day of euthanasia were included in the analysis: the Cancer group ($n = 7$) and the Cancer + Ex group ($n = 6$) (Figure 1a). The research was conducted according to national and international ethical standards; the animal protocol was approved by the Ethics Committee on Animal Use (CEUA) of the School of Technology and Sciences (FCT/UNESP), under protocol number 02/2020.

4.2. Chemical Cancer Induction

At 50 days of age (week 0), the Cancer groups received a single intraperitoneal injection of 7,12-dimethylbenz[a]anthracene (DMBA) (Sigma Aldrich®, St. Louis, MO, USA) at a dose of 65 mg/kg of body weight, dissolved in sesame oil [39,40] (Figure 1a).

4.3. Incremental Load Test and Aerobic Physical Training Protocol

At week 13, the Cancer + Ex group was acclimated to aerobic exercise using an Inbramed® treadmill (Presidente Prudente, São Paulo, Brazil). The animals were submitted to an incremental load test to determine each animal's maximum power. The incremental load test began at 6 m/min with a 0% incline, increasing by 3 m/min every 3 min until the rats reached voluntary exhaustion, defined as touching the end of the treadmill five times within one minute. The maximum power (Pmax), representing the speed at exhaustion (m/min), was used to tailor the training intensities. Animals were subjected to three tests during the protocol period to adjust for overload: first, before the 1st week of physical training (week 13); second, at the 4th week (week 17) of physical training (midpoint), and 8th week (week 21) of physical exercise (endpoint). Using this personalized data, we set an

overload of 60% of the maximum speed, deemed moderate in intensity for aerobic physical exercise performance. The running time was reduced by 20 min when the animal failed to exercise the total target duration. Most animals ran only 20 min in the last three weeks due to progressive exercise intolerance. The chronic aerobic exercise protocol was based on the study by Ferreira et al. [41] and consisted of 8 weeks. Each experimental week consisted of 5 consecutive training sessions and two days of rest. The first week consisted of 15 min of exercise duration, and it was increased to 20 min in the second week, 30 min in the third week, 35 min in the fourth week, 40 min in the fifth week, 45 min in the sixth week, 50 min in the seventh week, and 60 min in the eighth week.

4.4. Tissues Collection

At week 21, the rats were weighed with an analytical balance (Bell, Piracicaba, São Paulo, Brazil). Subsequently, the rats were euthanized via decapitation, in strict adherence to CONCEA Normative Resolution number 37/2018—Euthanasia Practice Guideline of the National Council for the Control of Animal Experimentation. After euthanasia, the muscles, liver, pancreas, kidney, adipose tissue, and tumors were dissected, weighed, and snap-frozen in liquid nitrogen. The serum was collected and stored at -20°C . All sample weights (except tumor weights) were normalized to the initial body weight (IBW) to control differences in body size at the start of the study. Tumor weight was normalized to the final body weight and expressed as a percentage (%).

4.5. Tumor Volume

The tumor volume was assessed at the end of the study using a caliper. We measured the individual mice's tumors (width and length) and then calculated the tumor volume using the equation $[V = (W^2 \times L)/2]$ suggested by Faustino-Rocha et al. [42], where V = volume, W = width, and L = length. The results are presented in Table S1.

4.6. Food Intake Measurements

The food intake was assessed twice a week. The remaining chow was weighed and replenished up to 500 g. The food intake was assessed every 3–4 days.

4.7. Histological Analysis

Tumors and the tibialis anterior muscle were collected, weighed, and fixed in 10% buffered formalin for 24 h. After fixation, the tissues were washed in 70% ethyl alcohol, with subsequent dehydration in an increasing series of alcohol dilutions, embedded in paraffin and plastic polymer (Paraplast Plus ST, St. Louis, MO, USA), and sectioned to a thickness of $5\text{ }\mu\text{m}$ and stained with hematoxylin-eosin (H&E) for general morphology (tumor and tibialis anterior), and picrosirius red in tibialis anterior for analysis of collagen deposition. Images were photographed using an Axiophot photomicroscope (Zeiss, Munich, Germany) and AxioVision Rel software 4.8, at $400\times$ magnification. Tibialis anterior frozen sections were made ($10\text{ }\mu\text{m}$) using a Microm (HM-505-E) cryostat (MICROM International GmbH, model HM-505-E, Walldorf, Germany), followed by the nicotinamide adenine dinucleotide tetrazolium reductase staining to quantify the Cross-Sectional Area (CSA). After staining, four pictures were taken randomly at $100\times$ magnification, and then the CSA was quantified using ImageJ software (version 1.54p). The average of those 4 images was included in the final analysis. The mice that did not develop tumors or whose section quality was low were excluded from the analysis.

4.8. Redox State Biomarker Analysis in the Tibialis Anterior Muscle

The tibialis anterior muscle was thawed on ice and rinsed with ice-cold saline solution (0.9% NaCl, *w/v*). Homogenates were prepared using a Rotor-Stator TH homogenizer

(Omni International, Kennesaw, GA, USA) operating at medium speed for 90 s to obtain 5% (*w/v*) tissue homogenates in 50 mmol/L sodium phosphate buffer (pH 7.4) supplemented with 0.2% (*v/v*) Triton X-100, 10 mmol/L diethylenetriaminepentaacetic acid (DTPA), and 2 mmol/L phenylmethanesulfonyl fluoride (PMSF). The homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C using an Eppendorf R 5810 centrifuge (Eppendorf, Hamburg, Germany). The supernatants were collected, aliquoted, and stored at −80 °C for subsequent analyses. For reduced glutathione (GSH) quantification, tissue homogenates were prepared in 5% (*w/v*) trichloroacetic acid containing 2 mmol/L DTPA and centrifuged at 4,000 rpm for 10 min at 4 °C. Oxidative damage markers were assessed by evaluating lipid peroxidation through thiobarbituric acid-reactive substances (TBARS) formation [43] and protein oxidation via protein carbonyl quantification using the 2,4-dinitrophenylhydrazine (DNPH) alkaline method [44]. Non-enzymatic antioxidant defenses were evaluated by measuring the total antioxidant capacity using the ferric reducing antioxidant power (FRAP) assay [45] and estimating GSH levels with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) [46]. Enzymatic antioxidant defenses were assessed by determining superoxide dismutase (SOD) activity, based on the inhibition of pyrogallol autoxidation [47], and catalase activity, calculated from the rate of hydrogen peroxide (H₂O₂) decomposition [48]. All results were normalized to total protein content and quantified using the Hartree-Lowry method [49]. All analyses were conducted in the Saliva Biochemistry Laboratory (LABIQS) at the School of Dentistry, Araçatuba—FOA/UNESP.

4.9. RNA Sequencing

RNA was isolated from ~50 mg of frozen tibialis anterior muscle and tumor tissue via the RNeasy Mini Kit (Qiagen, Hilden, Germany). The concentration and quality of total RNA samples were assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany), and an RNA Integrity Number (RIN) of eight or higher was required to pass the quality control. We isolated RNA from four animals per group. We sent it for library preparation and sequencing to the Massively Parallel Sequencing Shared Resource at Oregon Health and Science University (OHSU) using the Illumina NovaSeq6000 (Illumina, Inc. San Diego, CA, USA). 150 ng of total RNA was used for each sample. cDNA library preparation included mRNA purification/enrichment, RNA fragmentation, cDNA synthesis, and ligation of index adaptors using the KAPA mRNA Hyperprep Kit prep (KK8581, Roche, Indianapolis, IN, USA). Each resulting indexed library was quantified, and its quality was assessed by NovaSeq 6000. 5 µL of 2 nM pooled libraries per lane was denatured, neutralized, and applied to the cBot for flow cell deposition and cluster amplification before loading to 100 bp paired-end sequencing (Illumina, Inc., San Diego, CA, USA). Approximately 30–50 million reads per library were generated. A Phred quality score (Q score) was used to measure sequencing quality. The reads were mapped to the UCSC *Rattus norvegicus* (rn7) reference genome using STAR (Spliced Transcripts Alignment to a Reference). To evaluate the quality of the RNA-seq data, the number of reads that fell into different annotated regions (exonic, intronic, splicing junction, intergenic, promoter, UTR, etc.) of the reference genes was determined with bamUtils 0.5.9. Low-quality mapped reads (including reads mapped to multiple positions) were excluded, and featureCount 1.5.1 was used to quantify the gene-level expression. Differential gene expression analysis was determined using DESeq2. Those proceedings were performed using Partek™ Flow™ software, v11.0. The PCA for all expressed genes was built using R (<https://www.r-project.org/> (accessed on 12 April 2024)) and RStudio (<https://posit.co/download/rstudio-desktop/> (accessed on 12 April 2024)), using the packages ggplot2, dplyr, factoextra, ggpubr, FacToMineR, and matrixStats. Differentially expressed genes (those with fold change >|2| and false discovery rate (FDR) < 0.05) were visualized with Morpheus (Morpheus,

<https://software.broadinstitute.org/morpheus> (accessed on 12 April 2024)). Volcano plots were generated with VolcanoR (VolcanoR—Exploring volcano plots (uva.nl), <https://huygens.science.uva.nl/VolcanoR/> (accessed on 12 April 2024)). All enrichment analysis was performed using EnrichR, filtering on two libraries (GO Biological Process 2023 and Reactome 2022) (Enrichr (<https://maayanlab.cloud/Enrichr/> (accessed on 12 April 2024))). The differentially expressed genes (DEGs) were also uploaded to the web tool Intervene to perform an UpSet Plot (Intervene—an interactive Shiny app for UpSet plots, Venn diagrams, and pairwise heatmaps (shinyapps.io), <https://shiny.hiplot.cn/intervene-shiny/> (accessed on 12 April 2024)). Meta-analysis of tibialis DEGs was made using the Illumina BaseSpace Correlation Engine. To interrogate the possible autocrine and paracrine communication between the tumor and tibialis anterior, we first filtered the genes for ligands and receptors [50], selected all with FC > 2 and *p* value < 0.05, and calculated the communication score [51]. The alluvial plot was generated using SankeyMATIC (<http://sankeymatic.com/> (accessed on 12 April 2024)).

4.10. Immune Cell Population

The deconvolution analysis and abundance of 22 types of immune cells were performed by uploading the entire tibialis anterior and tumor transcriptome into CIBERSORTx (<https://cibersortx.stanford.edu/> (accessed on 12 November 2024)). We exported the CIBERSORTx data and imported it into GraphPad Prism v.10 to run the statistical analysis.

4.11. Tumor Identity

To characterize the tumor type caused by I.P. injection of DMBA, we ranked the 1000 most expressed genes by mean expression. Subsequently, we performed an enrichment analysis using EnrichR (Enrichr (maayanlab.cloud)), employing the ARCHS4 Tissues and Azimuth Cell Types 2021 libraries for ranking. We interrogated markers of sarcoma as described in Parham et al. [11], including *Acta1* (Actin Alpha 1, Skeletal Muscle), *Ckm* (Creatine Kinase, M-type), *Des* (Desmin), *Mb* (Myoglobin), *Myh1* (Myosin Heavy Chain 1), *Myh10* (Myosin Heavy Chain 10), *Myh11* (Myosin Heavy Chain 11), *Myh2* (Myosin Heavy Chain 2), *Myh3* (Myosin Heavy Chain 3), *Myh4* (Myosin Heavy Chain 4), *Myh7* (Myosin Heavy Chain 7), *Myh9* (Myosin Heavy Chain 9), *Myl1* (Myosin Light Chain 1), *Myl11* (Myosin Light Chain 11), *Myl12a* (Myosin Light Chain 12A), *Myl12b* (Myosin Light Chain 12B), *Myl3* (Myosin Light Chain 3), *Myl4* (Myosin Light Chain 4), *Myl6* (Myosin Light Chain 6), *Myl9* (Myosin Light Chain 9), *Myod1* (Myogenic Differentiation 1), *Myog* (Myogenin), to confirm the classification of rhabdomyosarcoma.

4.12. Proteome Profile Array

Serum samples were collected, centrifuged, and kept on ice throughout handling, and stored at −80 °C until proteomic analyses were performed. A total of 79 cytokines were analyzed using the Proteome Profiler Rat XL Cytokine Array Kit (ARY030; R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. We used five animals per group, pooling 50 µL of serum from each animal, totaling 250 µL per group in duplicate. We homogenized the tumor in liquid nitrogen and extracted proteins from 100 mL RIPA buffer (89900; Thermo Fisher, Rockford, IL, USA) with Protease Inhibitor (1861280; Thermo Fisher) from 10 mg of tissue. Protein concentration was measured by Pierce BCA Protein Assay Kit (23227; Thermo Fisher). In each group (Cancer = 4 tumors and Cancer + Ex = 5 tumors), we pooled 200 µg of protein and analyzed it in duplicates. The dot of each blot was analyzed by densitometry using ImageJ-Fiji software (National Institute of Health, Bethesda, MD, USA, v1.54g).

4.13. Statistical Analysis

Results are expressed as mean $\pm$ standard deviation. Upon validation by the Shapiro–Wilk normality test, we used one-way ANOVA, followed by Tukey’s post-test. We used an unpaired t-test for comparisons of the Cancer + Ex vs. Cancer groups. Correlations were calculated using Pearson’s correlation coefficient test. Significance was determined as $p < 0.05$. Analyses were performed using GraphPad Prism 10 software for Windows (version 10.4.1).

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Informed Consent Statement: Not applicable.

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