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Cancer cachexia decreases specific force and accelerates fatigue in limb muscle

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ABSTRACT

Cancer cachexia is a complex metabolic syndrome that is characterized by the loss of skeletal muscle mass and weakness, which compromises physical function, reduces quality of life, and ultimately can lead to mortality. Experimental models of cancer cachexia have recapitulated this skeletal muscle atrophy and consequent decline in muscle force generating capacity. However, more recently, we provided evidence that during severe cancer cachexia muscle weakness in the diaphragm muscle cannot be entirely accounted for by the muscle atrophy. This indicates that muscle weakness is not just a consequence of muscle atrophy but that there is also significant contractile dysfunction. The current study aimed to determine whether contractile dysfunction is also present in limb muscles during severe Colon-26 (C26) carcinoma cachexia by studying the glycolytic extensor digitorum longus (EDL) muscle and the oxidative soleus muscle, which has an activity pattern that more closely resembles the diaphragm. Severe C-26 cancer cachexia caused significant muscle fiber atrophy and a reduction in maximum absolute force in both the EDL and soleus muscles. However, normalization to muscle cross sectional area further demonstrated a 13% decrease in maximum isometric specific force in the EDL and an even greater decrease (17%) in maximum isometric specific force in the soleus. Time to peak tension and half relaxation time were also significantly slowed in both the EDL and the solei from C-26 mice compared to controls. Since, in addition to postural control, the oxidative soleus is also important for normal locomotion, we further performed a fatigue trial in the soleus and found that the decrease in relative force was greater and more rapid in solei from C-26 mice compared to controls. These data demonstrate that severe cancer cachexia causes profound muscle weakness that is not entirely explained by the muscle atrophy. In addition, cancer cachexia decreases the fatigue resistance of the soleus muscle, a postural muscle typically resistant to fatigue. Thus, specifically targeting contractile dysfunction represents an additional means to counter muscle weakness in cancer cachexia, in addition to targeting the prevention of muscle atrophy.

1. Introduction

Cachexia is a complex metabolic syndrome associated with most advanced stage cancers that is characterized by progressive weight loss due to significant skeletal muscle wasting, with or without adipose tissue wasting [1]. The muscle wasting causes associated muscle weakness and cancer patients have shown 33–40% reductions in quadriceps muscle strength compared to healthy volunteers [2]. This weakness compromises physical function, functional independence and quality of life. Experimental models of cancer cachexia have recapitulated this limb muscle weakness, with several groups independently reporting a decrease in maximal tetanic force in the EDL and TA muscles [3–5]. These muscles are commonly studied during cancer cachexia because cachexia is known to affect glycolytic muscles to a greater extent than oxidative muscles [6–9]. Interestingly, in each of these studies specific force (force normalized to cross sectional area) was not different between control and C-26 mice, suggesting that the muscle weakness is due entirely to the decrease in muscle mass. Yet recently published data from our lab [10] and another [5] clearly demonstrate a decrease in diaphragm specific force during severe cancer cachexia. These combined findings suggest heterogeneity in skeletal muscle's response to cancer cachexia, with a decrease in the specific force of some skeletal muscles, such as the diaphragm, but not others, such as the EDL and TA muscles. This could be related to muscle use, in that the diaphragm contracts continuously

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whereas the EDL and TA muscles contract significantly less frequently. Therefore the aim of the current study was to determine whether severe cancer cachexia causes contractile dysfunction in the oxidative soleus muscle, a postural limb muscle that is chronically active and functionally more similar to the diaphragm. For comparative purposes we also measured contractile properties in the glycolytic EDL muscle.

2. Materials and methods

2.1. Animals

Male CD2F1 mice (~20 g) purchased from Charles River Laboratories (Wilmington, Massachusetts) were used for all animal experiments, and all animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee. Mice were provided with water and standard diet *ad libitum*. Mice were kept in a controlled facility on a 12 h light–dark cycle.

2.2. Cancer cachexia

Colon-26 (C-26) cells were obtained from the National Cancer Institute Tumor Repository (Frederick, MD, USA). These cells were cultured in RPMI 1640 (Mediatech, Herndon, VA, USA) supplemented with 10% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin at 37 °C in a 5% CO_2 humidified atmosphere. Severe cancer cachexia was induced by subcutaneous injection of 5×10^5 cells into each flank, and muscle were removed for analysis when the largest tumor reached 1.5 cm in diameter (~25–27 days).

2.3. Muscle preparation and analysis

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg bodyweight) and the soleus and extensor digitorum longus (EDL) muscles were dissected once a surgical level of anesthesia was reached. Solei and EDL muscles used for *in vitro* contractile measurements were used immediately, as described below. For histological analysis, separate solei and EDL muscles were embedded in freezing medium in a tissue-embedding cassette prior to freezing in liquid-nitrogen-cooled isopentane, and stored at −80 °C.

2.4. In vitro muscle contractile properties

The solutions and methods used for studies of limb muscle isometric function were described in detail previously [11–13]. Briefly, mice were anesthetized using isoflourane (5%, induction; 3% maintenance), and the muscle of interest excised and immediately placed in buffer solution for dissection. One end of the muscle was tied to a Dual-Mode Muscle Lever System (300C-LR, Aurora Scientific Inc, Aurora, Canada) and the other end onto a secured glass rod using a 4.0 gauge silk suture. Muscles were placed at optimal length (Lo) and allowed 20 min of thermo-equilibration to the desired temperature (32 °C). Thereafter, measurements of force–frequency were initiated. In all electrical stimulations, a supramaximal current (600–800 mA) of 0.25 ms pulse duration was delivered through a stimulator (701C, Aurora Scientific Inc.), while train duration for isometric contractions was 300 ms for the EDL and 500 ms for the soleus. The soleus muscle was stimulated to fatigue during isometric contractions (pulse frequency 40 Hz, train duration 500 ms, train rate 0.25 Hz). All data were recorded and analyzed using commercial software (DMC and DMA, Aurora Scientific). Specific force (N/cm^2) of EDL and soleus muscles were multiplied to the ratio of fiber length to muscle length published previously [14].

2.5. Immunohistochemistry

Sections (10 μm) were taken from the midbelly of the soleus and EDL muscles using a Microm HM 550 cryostat (Microm International, Walldorf, Germany) and processed for identification of the different muscle fiber types, as recently described [10], using primary antibodies for myosin heavy chain (MHC) Type I (A4.840; Developmental Studies Hybridoma Bank, Iowa City, IA USA), MHC Type IIa (SC-71; Developmental Studies Hybridoma Bank), and laminin (Sigma Aldrich, St. Louis, MO, USA). Images were visualized and captured using a Leica DM5000B microscope (Leica microsystems, Bannockburn, IL, USA) and muscle fiber cross sectional area (CSA) traced using Leica Application Suite 3.5.0 software.

2.6. Statistical analysis

All data were analyzed using a Student's *t* test or a 2-way ANOVA followed by Bonferroni post hoc comparisons (GraphPad Software, San Diego, CA). All data are expressed as means ± SE, and significance was set at $P \leqslant 0.05$.

3. Results

In agreement with data recently published from our lab, C-26 mice showed a significant decrease in body weight at the study endpoint when tumor diameter reached 1.5 cm (25% decrease; data not shown). This decrease in body weight was accompanied by a significant 20% decrease in the soleus (control, 8.9 mg ± 0.4; C-26, 6.3 mg ± 0.4) and a 27% decrease in the EDL (control, 9.9 mg ± 0.3; C-26, 7.8 mg ± 0.6) muscle mass.

3.1. Soleus muscle contractile dysfunction and muscle fiber atrophy

The absolute force–frequency relationship of the soleus muscle is displayed in Fig. 1A and shows that submaximal and maximal isometric tetanic force were decreased by 25–31% in C-26 mice compared to controls (Fig. 1B). We subsequently normalized the force to physiological cross sectional area (Fig. 1C) and found that maximum specific force was significantly decreased by 17% (control, 29.19 ± 1.24 N/cm^2; C-26, 24.37 ± 0.69 N/cm^2; Fig. 1C and D), demonstrating that the soleus muscle weakness cannot be explained by a decrease in muscle mass alone. Time to peak tension and half relaxation time were also significantly greater in the solei of C-26 mice compared to controls (Fig. 1E and F). Because the soleus muscle is postural and used during ambulatory activities and thus "built" to be fatigue-resistant, we also measured muscle force production during a fatigue trial. The decrease in relative force (as a percentage of initial force) was greater and more rapid, indicative of less fatigue resistance, in solei from C-26 mice compared to controls (Fig. 1G). Thus, relative force was significantly lower in C-26 mice at 300 and 600 s compared to controls (Fig. 1H).

Although the soleus muscle atrophies during severe cancer cachexia, as shown here and by others [15], we performed immunohistochemistry to identify muscle fiber types and then determined the mean fiber cross sectional area of type I, type IIa and type IIb/x fibers in control and C-26 mice. The mean fiber cross sectional area of type IIa and type IIb/x fibers were significantly decreased by 21% and 26%, respectively, in the C-26 group compared to controls (Fig. 1I and J). However, the 15% decrease in mean CSA of Type I fibers from C-26 mice did not reach statistical significance. We also quantified the proportion of each muscle fiber type to determine whether, in our hands and model, C-26 cancer cachexia causes a muscle fiber type shift. Indeed, we found a 26% decrease in the percentage of type I fibers and a 20% increase in the percentage of type

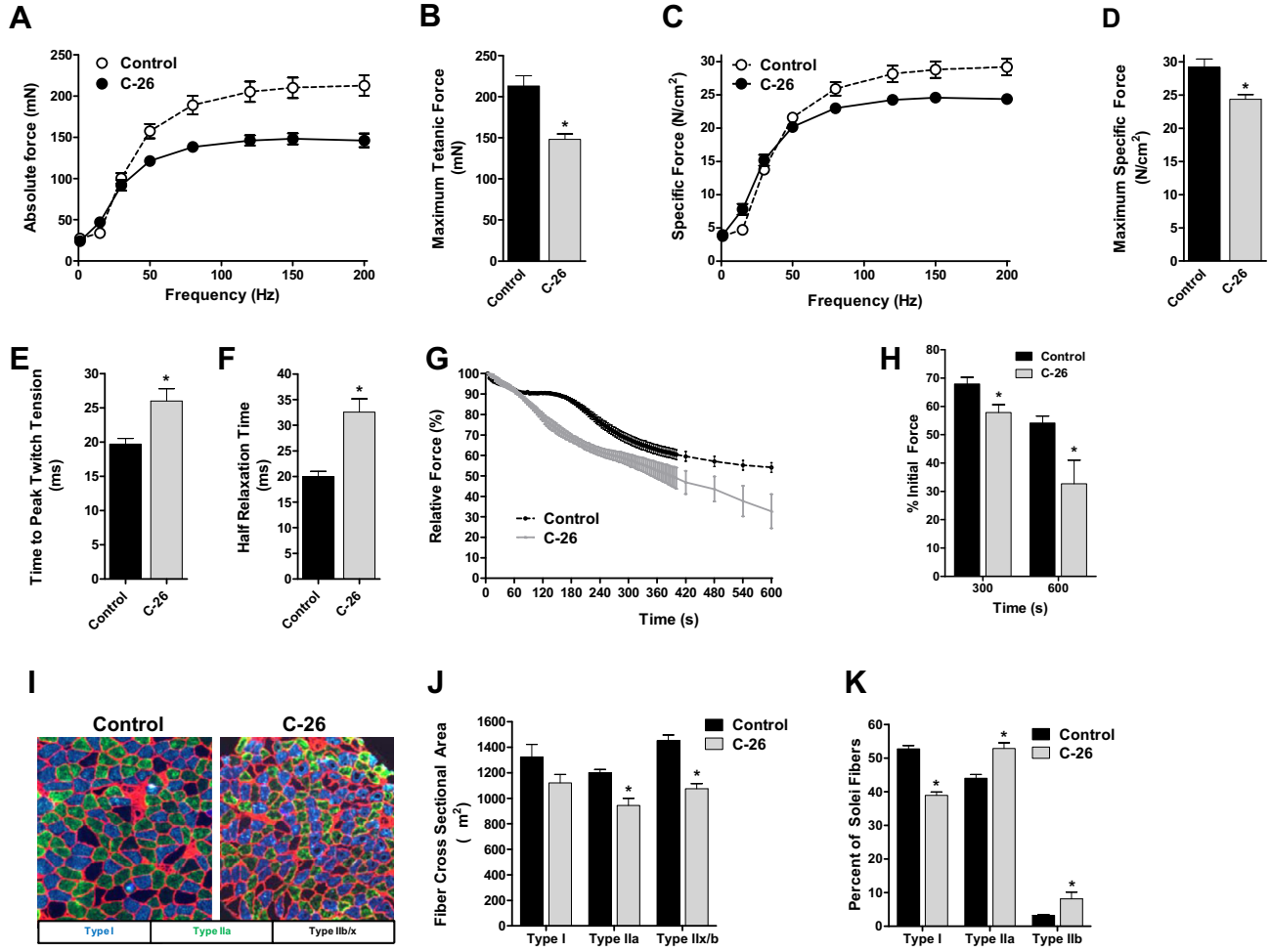

Fig. 1. Soleus contractile properties and fiber size in control and C-26. (A) The absolute force-frequency relationship, (B) maximal tetanic force, (C) specific force–frequency relationship, (D) maximum specific force, (E) time to peak twitch tension, (F) one half twitch relaxation time, (G) relative force (in percent of initial force) during fatigue protocol, and (H) percent of initial force at select time-points. (I) Representative images taken from cross sections of the soleus in control and C-26 mice. Sections were incubated with an anti-laminin antibody to allow for visualization of muscle fibers (red), and anti-myosin heavy chain (MHC) Type I (blue) and anti-MHC Type IIa (green) antibodies. Black fibers represent Type IIb/x fibers. The cross sectional area (CSA) of each muscle fiber type was measured and the mean CSA of each fiber type shown in (J). (K) Percentage of each soleus muscle fiber type in control and C-26 mice. Each bar represents the mean ± SE for 6 muscles per group. *Significantly different from control ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

IIa fibers in the solei of C-26 mice compared to controls (Fig. 1K). The percentage of type IIb/x fibers also significantly increased in the solei of C-26 mice, but these fibers represent a very small fraction of the soleus fiber type distribution (control, 3.3% type IIb/x fibers; C-26, 8.1% type IIb/x fibers).

3.2. EDL muscle contractile dysfunction and muscle fiber atrophy

Although the primary purpose was to determine whether severe cancer cachexia causes contractile dysfunction in the oxidative soleus muscle, for comparative purposes we also tested the glycolytic EDL muscle. The absolute force-frequency relationship of the EDL muscle is shown in Fig. 2A and clearly shows that submaximal and maximal absolute force were decreased by 30–35% in severely cachectic C-26 mice compared to controls. Indeed, the maximum isometric tetanic force was decreased by 33% in C-26 compared to controls (Fig. 2B). Similar to the soleus muscle, this weakness in the EDL cannot be explained by a decrease in muscle mass alone since normalization to muscle cross sectional area, to generate specific force, revealed deficits that remained in C-26 mice compared to controls (Fig. 2C). Indeed, maximum specific force was significantly decreased by 13% (control, 27.07 ± 0.64 N/cm^2; C-26 23.48 ± 0.96 N/cm^2; Fig. 2D). The contraction (time to peak twitch tension) and half relaxation times were also significantly prolonged in C-26 mice compared to controls (Fig. 1E and F).

In addition to measuring *in vitro* muscle contractile function, we also quantified the magnitude of fiber atrophy in each of the muscle fiber types of the EDL. Muscle fiber size was significantly decreased in both type IIa and Type IIb/x fibers of C-26 mice compared to controls, by 38% and 40%, respectively (Fig. 2G and H). We also quantified the EDL muscle fiber type distribution to determine if, like the soleus, C-26 cancer cachexia induces a fiber type shift. Similar to the findings in the soleus muscle, C-26 mice showed a fiber type shift towards type IIb/x fibers, with the percentage of type IIa fibers decreasing from ~30% to ~17% and the percentage of type IIb/x fibers increasing from ~70% to ~83% (Fig. 2I).

4. Discussion

The significant skeletal muscle wasting associated with cancer cachexia causes profound muscle weakness, with cancer patients showing 33–40% reductions in quadriceps muscle strength compared to healthy volunteers [2]. This magnitude of muscle weakness has substantial effects on physical function, functional independence and quality of life. To date only a handful of studies

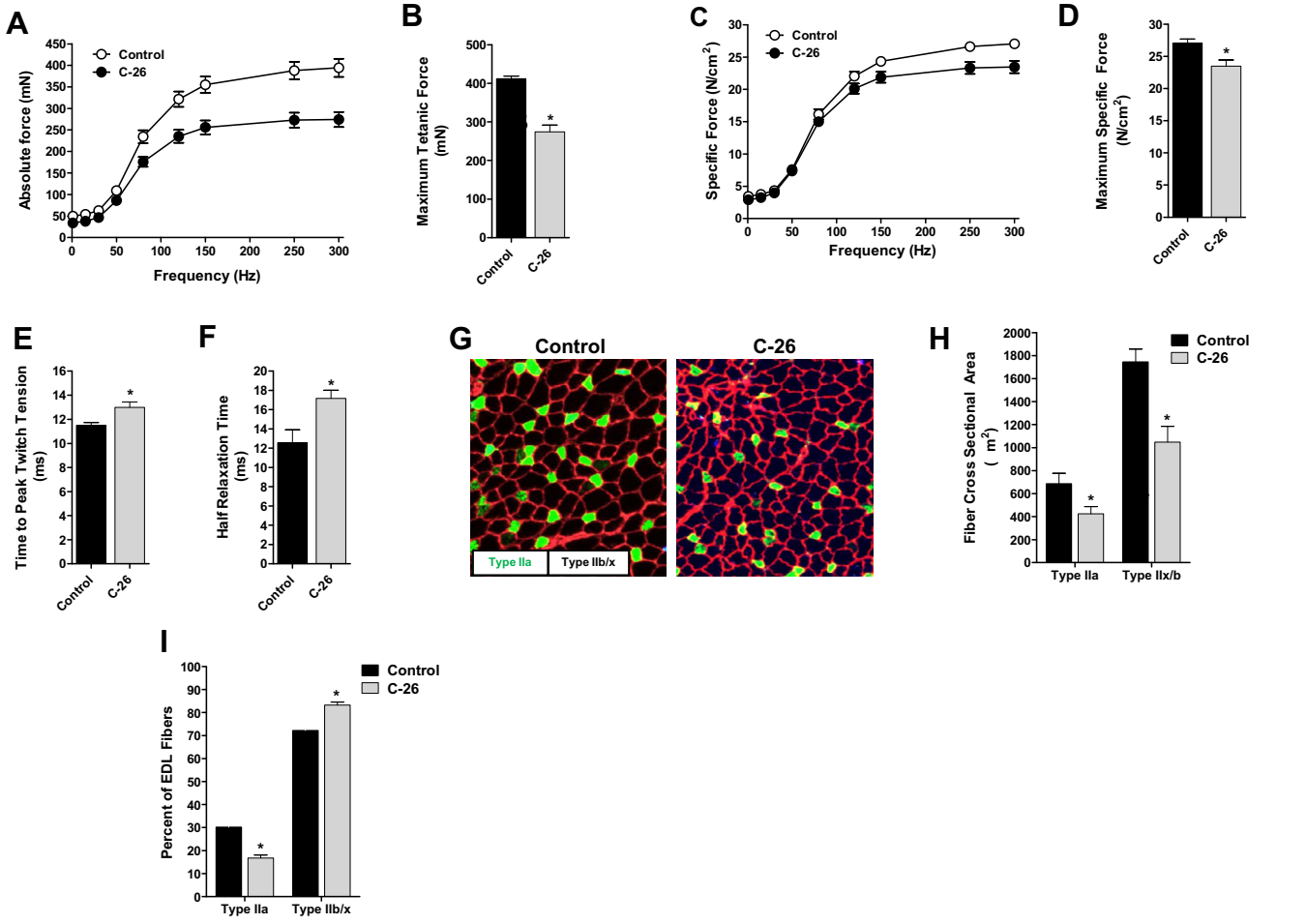

Fig. 2. Extensor digitorum longus contractile properties and fiber size in control and C-26 tumor bearing mice. (A) The absolute force-frequency relationship, (B) maximal tetanic force, (C) specific force–frequency relationship, (D) maximum specific force, (E) time to peak twitch tension and, (F) one half twitch relaxation time of the EDL from control and ~26 day C-26 tumor bearing mice. (G) Representative cross sections taken from the EDL of control and C-26 mice. Sections were incubated with an anti-laminin antibody to allow for visualization of muscle fibers (red), and anti-myosin heavy chain (MHC) Type I (blue, but no Type I fibers were detected) and anti-MHC Type IIa (green) antibodies. Black fibers represent Type IIb/x fibers. The cross sectional area (CSA) of each muscle fiber type was measured and the mean CSA of each fiber type shown in (H). (I) Percentage of each EDL muscle fiber type in control and C-26 mice. Each bar represents the mean ± SE for 6 muscles per group. *Significantly different from control ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

have recapitulated this muscle weakness in experimental models of cancer cachexia. Studies in the EDL and TA muscles clearly show a significant decrease in maximum force production, but attribute this weakness entirely to the decrease in muscle mass such that maximal specific force is not different in muscles of control and cachectic animals [3–5,15]. However two recent studies in the diaphragm show a significant decrease in specific force during cancer cachexia [5,10]. Thus, in the diaphragm muscle weakness is due not only to muscle fiber atrophy but also to compromised excitability of the sarcolemma, calcium release, and/or myofibrillar proteins. These findings suggest there is heterogeneity in skeletal muscle's response to cancer cachexia, which is surprising given the systemic nature of cancer. In the current study we aimed to determine the effect of cancer cachexia on the functional response of a locomotor muscle with an activity pattern more similar to the diaphragm than the EDL and TA. We found a significant decrease in maximal tetanic force in the soleus muscle of C-26 mice compared to control, which is in agreement with previous studies in EDL and TA muscles. However, we also found a significant 17% decrease in the maximal specific force of the solei from C-26 mice compared to controls. Whilst substantially less than the 30% decrease in maximal tetanic force, this finding suggests that the decrease in maximal force cannot be explained entirely by the muscle atrophy and further suggests that interventions should not only focus on preserving muscle mass, but also focus on means to protect the contractile apparatus. Moreover, the soleus muscle of C-26 mice also showed increased fatigability compared to controls, and prolonged contraction and relaxation times, further demonstrating and substantiating contractile deficits in this muscle. Given that the soleus muscle is a postural muscle that is active during standing and walking, its weakness and increased fatigability due to cancer cachexia may play a significant role in compromising physical function.

We subsequently determined the contractile properties of the EDL muscle to identify whether our model of severe cancer cachexia also negatively affects the contractile apparatus in a glycolytic locomotor muscle, which in previous studies has not shown statistically significant deficits in specific force [3,15]. In agreement with these previous studies, maximal tetanic force was significantly decreased in the EDL of C-26 mice. However, unlike the findings of others, but similar to our findings in the soleus, maximal specific force of the EDL was also significantly decreased by 13% in C-26 mice compared to controls. The decrease in soleus and EDL muscle specific force suggests that, in our model of severe cancer cachexia, the muscle weakness is caused not only by muscle atrophy but also by additional factors which negatively regulate excitation–contraction coupling and development and transmission of force. In support of this, electron micrographs have clearly shown significant

disorganization of myofibrils in the limb muscles of mice during cancer cachexia [7,15,16] which would presumably cause significant contractile deficits.

The significant fiber type shift in the soleus from type I fibers towards type II fibers is in agreement with previously published work [17]. However, this fiber type shift in both the soleus and EDL cannot explain all of the contractile changes reported in the current study. Indeed, C-26 caused a decrease in specific force and slower contraction and relaxation times, which would be unexpected during a shift towards type II fibers since specific force is higher and contraction/relaxation times faster in type II fibers. In fact the only functional outcome that could be explained by the fiber type shift is the decreased relative force during the fatigue trial in the soleus (Fig. 1G and H) since type II fibers are less fatigue resistant.

In summary our findings in the present study show that, in addition to glycolytic muscles, the oxidative soleus muscle is significantly atrophied, weaker and less fatigue-resistant in mice with cancer cachexia compared to controls. Our findings also show, for the first time, that although locomotor muscle atrophy during severe C-26 cancer cachexia accounts for a significant proportion of the muscle weakness, additional factors negatively affecting contractile function are also present during cancer cachexia. This suggests that countermeasures aiming to prevent muscle weakness associated with cancer cachexia should target pathways that lead to muscle atrophy as well as those which contribute to contractile dysfunction.

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