Journal of Visualized Experiments

Mouse models of cancer-induced cachexia: hind limb muscle mass and evoked force as readouts

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE57247R2
Full Title:	Mouse models of cancer-induced cachexia: hind limb muscle mass and evoked force as readouts
Keywords:	cancer-induced cachexia; skeletal muscle wasting; magnetic resonance imaging (MRI); evoked force
Corresponding Author:	Shinji Hatakeyama Novartis Pharma AG Basel, SWITZERLAND
Corresponding Author's Institution:	Novartis Pharma AG
Corresponding Author E-Mail:	shinji.hatakeyama@novartis.com
First Author:	Marie Jourdain
Other Authors:	Marie Jourdain
	Stefan Melly
	Serge Summermatter
Author Comments:	
Additional Information:	
Question	**Response**
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Dear Editor,

Please find enclosed a manuscript by Jourdain et al. entitled “Mouse models of cancer-induced cachexia: hind limb muscle mass and evoked force as readouts”. We kindly ask the manuscript to be considered for publication in *Journal of Visualized Experiments* as a research article. Cachexia associated with advanced cancer is a severe symptom reducing quality of life and life expectancy of cancer patients. Currently, no effective therapies are available to treat cachexia.

We describe here detailed protocols to generate and evaluate two murine models of cancer-cachexia, one syngeneic and one xenograft. Furthermore, readouts such as Magnetic Resonance Imaging (MRI) and evoked force measurement that are used to evaluate muscle mass, volume and function are described. This protocol will allow the testing of potential therapeutic agents for the treatment of cachexia.

We hope you agree that this study is appropriate for consideration in *Journal of Visualized Experiments*.

Best regards,

Shinji Hatakeyama, Ph.D.

TITLE:

Mouse Models of Cancer-induced Cachexia: Hind Limb Muscle Mass and Evoked Force as Readouts

AUTHORS AND AFFILIATION:

Jourdain Marie, Melly Stefan, Summermatter Serge, Hatakeyama Shinji

MusculoSkeletal Diseases, Novartis Institutes for Biomedical Research
Novartis Pharma AG, CH-4002 Basel, Switzerland

EMAIL ADDRESSES OF CO-AUTHORS:

Jourdain Marie (marie.jourdain@novartis.com)
Melly Stefan (stefan.melly@novartis.com)
Summermatter Serge (serge.summermatter@novartis.com)
Hatakeyama Shinji (shinji.hatakeyama@novartis.com)

CORRESPONDING AUTHOR:

Hatakeyama Shinji (shinji.hatakeyama@novartis.com)

KEYWORDS:

cancer-induced cachexia; skeletal muscle wasting; magnetic resonance imaging (MRI); evoked force

SHORT ABSTRACT:

Progressive cachexia affects the majority of patients with advanced cancer. There is an urgent medical need for effective treatments. However, there is no specific therapeutics currently available. Here we describe mouse models and readouts for cancer-induced cachexia to allow the testing of potential therapeutic agents for the treatment of cachexia.

LONG ABSTRACT:

The majority of patients with advanced cancer suffer from cachexia, a systemic wasting syndrome, which causes a reduction in tolerance to anti-cancer treatment, response to therapy, quality of life, and eventually, duration of survival. Currently, there is no specific remedy available despite the urgent medical need for an effective treatment of cachexia. An important aspect of cachexia is the inexorable loss of skeletal muscle mass leading to progressive functional impairments. It is, therefore, crucial to identify early readouts for the loss of muscle mass and function to initiate appropriate and timely treatments. Here, we describe mouse models of cancer-induced cachexia using readouts such as hind limb muscle mass and volume, as well as evoked force and food intake measurement, to allow the testing of potential therapeutic agents for the treatment of cachexia. We focus on experimental cancer models using a mouse (syngeneic) or a human (xenograft) cancer cell lines with a rapid onset of tumor growth and cachexia, which are easier to establish, monitor and reproduce compared to the genetically engineered mouse models.

INTRODUCTION:

The majority of patients with advanced cancer suffer from cachexia, and cancer-induced cachexia results in a poor outcome, such as an increased toxicity to chemotherapy, a reduction in quality of life, and survival rate[1, 2]. It is proposed that skeletal muscle loss is the most prominent event in cancer cachexia and cannot be fully reversed by conventional nutritional support[3-5]. Despite the urgent need for the effective treatment of cancer cachexia, there is no specific therapeutic treatment currently available.

Various pre-clinical models have been developed to better understand the underlying mechanisms of muscle wasting associated with cancer and to explore the effectiveness of therapeutic strategies. Most of them involve the injection of cancer cell lines in immunocompetent (syngeneic) or immunosuppressed animals (xenograft)[6, 7]. Although many animal studies use bodyweight as an outcome, it is necessary to use additional dedicated readouts in order to characterize, more specifically, the loss of skeletal muscle mass and function.

We describe here detailed protocols to generate and evaluate two murine models of cancer-cachexia, one syngeneic[8-10], and one xenograft[9]. Furthermore, readouts such as Magnetic Resonance Imaging (MRI)[12] and evoked force measurement[13, 14] that are used to evaluate muscle mass, volume and function are described. The methods described here will enable testing and identification of potential therapeutic agents for the treatment of cancer cachexia. Furthermore, these readouts are widely applicable to other muscle wasting conditions such as other types of cachexia, sarcopenia and disuse-induced muscle atrophy.

PROTOCOL:

Protocols described here were performed according to the official regulations effective in the Canton of Basel-City, Switzerland, under the license number BS-2186. Animals under this license have to be euthanized if they experience a body weight loss of -20% or reach a tumor size of 1,500 mm^3. In experiments with CT-26 cells, mice typically reach the body weight loss criterion first. In experiments with A2058 cells, mice reach first the tumor volume criterion.

1. Cancer Cell Lines Preparation and Inoculation

Note: The mouse colon cancer cell line CT-26[15] was a gift (see **Table of Materials**). The human A2058 melanoma cell line was commercially purchased (see **Table of Materials**).

1.1. Mouse colon CT-26 cancer cell line

1.1.1. Thaw a frozen vial containing cells, transfer the cell suspension to a culture flask containing RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotic-antimycotic solution, and place at 37 °C with 5% CO_2.

1.1.2. After 2-3 passages performed every 2-4 days with a standard cell culture procedure before the cells become confluent, harvest CT-26 cells by treatment with a proteolytic cell detachment solution and suspend them in a solution containing 50% PBS and 50% of liquid basement membrane matrix in order to achieve a concentration of 3 x 10^6 cells/mL ensured by a standard electronic cell number counting method. Keep this cell suspension on ice to avoid polymerization of the hydrogel matrix.

1.2. Human A2058 melanoma cell line

1.2.1. Thaw a frozen vial containing cells and transfer the cell suspension to a culture flask containing DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotic-antimycotic solution and place at 37°C with 5% CO_2.

1.2.2. After 3-4 passages performed every 2-4 days with a standard cell culture procedure before the cells become confluent, harvest A2058 cells by the treatment with a proteolytic cell detachment solution and suspend them in a solution containing 50% PBS and 50% of liquid basement membrane matrix in order to achieve a concentration of 30-50 x 10^6 cells/mL ensured by a standard electronic cell number counting method. Keep this cell suspension on ice to avoid polymerization of the hydrogel matrix.

1.3. Cell inoculation

1.3.1. Habituate mice at least one week to the animal facility before performing cell inoculation. House them in groups of 5 or less animals at 25 °C with a 12:12 h light-dark cycle. Provide food and water *ad libitum*, typically use a standard laboratory diet containing 18.2% protein and 3.0% fat with an energy content of 15.8 MJ/kg.

Note: Use immunocompetent adult mice (BALB/c, at least 10-weeks old) for CT-26 cells inoculation. Perform A2058 human cells inoculation in immune-deficient mice (Athymic Nude, at least 10-week old). With immune-deficient animals perform all manipulations in a sterile environment.

1.3.3. Weigh the animal and put it under anesthesia with isoflurane mixed with O_2 as a carrier gas (induction at 4%, maintenance between 1.5 and 2%).

1.3.3. Apply protective gel to the eyes of the animal and shave the left or right flank.

1.3.4. Disinfect the shaved area with a solution containing 70% Ethanol or Betadine Solution.

1.3.5. Lift the chosen flank skin with forceps, and inject 0.1 mL of cell suspension containing 0.3 x 10^7 cells in case of CT-26 or 3-5 x 10^7 cells in case of A2058 subcutaneously into the left or right upper flank of a mouse using a syringe and a 24G needle. Inject 0.1 mL of 50% PBS and

50% of liquid basement membrane matrix into Non-tumor bearing control mice. Put the mouse back in its cage and monitor until it wakes up.

Note: The number of cells injected will ultimately result in substantial tumor growth while avoiding too rapid body weight loss.

2. Animal Monitoring

2.1. Measure the body weight and the tumor volume 2 to 3 times per week in the first 2 weeks and then every day until the end of experiment or as soon as mice lost more than 10% of their initial body weight.

2.2. Start measuring the tumor volume through the skin *in vivo* as soon as they become palpable, usually a few days after inoculation (ca. 3-5 days). Measure length and width using calipers and calculate the size of the tumor using the following formula: (length × width^2)/2.

2.3. Assess food consumption by weighing food 2-3 times per week throughout the experiment.

Note: Food intake is reported as the amount of food consumed, divided by the number of animals in the cage and the number of days.

2.4. Euthanize mice prematurely with CO_2 in the following cases: when body weight loss is close to 20%, tumor volume reaches 1,500 mm^3, when tumors become necrotic, or when the animal shows obvious and important signs of pain or discomfort.

2.5. At the end of the experiment, euthanize animals with CO_2, and collect and weigh tumor and skeletal muscles (*e.g.* tibialis anterior, gastrocnemius-soleus-plantaris complex and quadriceps).

3. Magnetic Resonance Imaging Measurement

3.1. Perform Magnetic resonance imaging (MRI) measurement under anesthesia with isoflurane at a concentration of 3%, using an MRI with 7 T / 16 cm wide-bore instrument equipped with a 9-cm inner-diameter actively shielded gradient insert.

3.1.1. Apply veterinary eye ointment to the eyes to avoid drying during the scan.

3.1.2. Put the mouse in the MRI animal bed in the supine position, stretch the legs gently and fix them with tape.

3.1.3. Maintain body temperature and monitor respiration throughout the experiment using a physiology monitoring system.

3.1.4. To define the area to be measured, switch on the laser and move the bed with the animal until the laser point in the middle between knee and ankle.

3.1.5. Place the bed into the magnet.

3.2. Measure muscle volume using a modified 3D FLASH (Fast Low-Angle SHot) sequence, and reconstruct the 3D water-only image with an imaging software.

3.2.1. Open the software, start a new study, enter and register experiment details. Choose three scans (1-TriPilot-multi, 2–Localizer, 3 - FLASH-IDEAL).

3.2.2. Start the first scan (1-TriPilot-multi), and click on the traffic light. Once the scan is completed, verify if the mouse is well positioned and centered for the next scan. Before to start the second scan (2–Localizer), select this scan and click on geometry editor and adjust the sagittal position window.

3.2.3. Once the scan is completed, select the scan 3 (FLASH-IDEAL) and click on geometry editor. Select load preferences, choose localizer and click on accept. To set up the final scan, click through the slices and adjust the axial position window.

3.2.4. Select the scan FLASH-IDEAL of the mouse to be analyzed in the paravison program, and then the water-only image will be reconstructed and visualized in the 3D window. Go through the slices to mark the knee and the ankle to define the range for imaging analysis.

3.2.5. Determine lower leg muscle volume by calculating the number of pixels in the water-only image within the range and using the geometry information to convert the pixels to a volume (mm^3).

4. *In Situ* Force Measurement

4.1. Position the animal on a heating plate to maintain its body temperature around 37 °C under anesthesia with isoflurane mixed with O_2 as a carrier gas (induction at 4%, maintenance between 1.5 and 2%).

4.2. Shave one leg, perform a ~1 cm incision parallel to the femur with small scissors and expose the sciatic nerve. Maintain it exposed using a glass hook, and prevent it from drying by embedding the nerve in electrode gel.

4.3. Expose the Achilles' tendon of the gastrocnemius-soleus-plantaris muscle complex, cut it loose together with a piece of a calcaneus and then attach it to a force displacement transducer using either a polyethylene or a metallic wire.

4.4. Attach the previously exposed sciatic nerve to a stimulating electrode and connect it to an electrical stimulator.

4.5. Immobilize knee and ankle joints with a clamp to avoid unwanted movements. Muscle and tendon have to be horizontally aligned with the force transducer.

4.6. Stimulate the sciatic nerve with square bi-phasic pulses of 0.5 ms duration at 20 mA. To evoke twitch contraction, apply 4 stimulations of 1Hz every 20 s were applied, and to evoke tetanic contraction, perform 11 stimulations ranging from 10 to 120 Hz every 30 seconds.

Note: The force of contraction produced by the gastrocnemius-soleus-plantaris muscle complex in response to electrical stimulation of the sciatic nerve is transmitted via a force displacement transducer, amplified by a bridge amplifier, acquired with a data acquisition system and analyzed using a dedicated software to obtain the evoked force parameters.

4.7. Once the stimulation protocol is finished, carefully detach the animal and sacrifice it immediately CO_2 before performing muscle dissection as described in 2.4).

REPRESENTATIVE RESULTS:

In the CT-26 tumor-bearing group, body weight decrease started around day 13 and was significantly decreased from day 15 when compared to the non-tumor group (**Figure 1A**). This was associated with a progressive tumor growth, with a mean tumor volume reaching ca. 800 mm^3 by the end of the study 3 weeks after cell inoculation. In animals inoculated with A2058 cells, bodyweight decrease, and tumor growth were slower as compared to CT-26 tumor-bearing mice (**Figure 1B**). Bodyweight was significantly decreased from day 17 post-inoculation, and the study was terminated 3 weeks after inoculation with a mean body weight loss of about 5% and a tumor volume of ca. 900 mm^3. A significant decrease of food intake was associated with body weight loss in both cancer models. It was present at earlier stages in A2058 tumor-bearing animals (**Figure 2B**) than in mice inoculated with CT-26 cell line (**Figure 2A**), although more pronounced in the latter.

CT-26 tumors induced a significant decrease in the pooled muscle weight by close to 20% as compared to the non-tumor bearing group (Sham; **Figure 3A**). Muscle atrophy appeared more pronounced in tibialis muscle (ca. 30%) and close to average in gastrocnemius complex or quadriceps. Muscle weight loss was milder in the A2058 tumor-bearing group (**Figure 3B**) when compared to CT-26, with a significant decrease in pooled muscle weight by about 10% as compared to the non-tumor bearing group (Sham). Muscle atrophy in this model appeared less pronounced in tibialis muscle when compared to gastrocnemius complex and quadriceps.

Calf muscle volume was assessed non-invasively using MRI in the CT-26 mouse colon cancer model only. Significant reduction in calf volume could be observed as early as seven days after cancer cells inoculation, in left (**Figure 4A**) and right leg (**Figure 4B**); hence prior to body weight loss. Calf muscle volume can, therefore, be used as an early, non-invasive marker to determine

the onset of muscle wasting in this model. Gastrocnemius complex isometric force was measured in response to sciatic nerve stimulation terminally at the end of both animal studies. The advantage of this method compared to other tests (e.g., grasping test) is that it is independent of the motivation of the animals and that it allows to assess the entire range of force production (from minimal to maximal force). In the CT-26 tumor-bearing group muscle force was significantly reduced as compared to sham animals for frequencies of stimulation as low as 40Hz (**Figure 5A**). In the A2058 tumor-bearing group this significant decrease could only be observed from 100 Hz (**Figure 5B**). Both muscle twitch (force at 1 Hz) and maximal force displayed were significantly decreased for the two cancer cachexia models.

FIGURE LEGENDS:

Figure 1: Changes in body weight and tumor volume. A) CT-26 mouse colon cancer and **B)** A2058 human melanoma -induced cachexia. Sham refers to non-tumor control animals that received the solution only (i.e. without cells). Body weight (including tumor weight) is expressed as % of change from the day of inoculation. Tumor length and width were measured with a caliper and tumor volumes were calculated using the formula length × width^2)/^{2}. Values are expressed as means ± SEM (n=10-11); *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.01$ versus non-tumor group by unpaired t-test comparison.

Figure 2: Progression of food intake. A) CT-26 mouse colon cancer and **B)** A2058 human melanoma -induced cachexia. Food intake was assessed by weighing food 2-3 times per week. Food intake is expressed as the weight in grams consumed by each mouse per day. Values are expressed as means ± SEM (n=10-11); *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.01$ versus non-tumor group by unpaired t-test paired comparison.

Figure 3: Muscles weight. A) CT-26 mouse colon cancer and **B)** A2058 human melanoma -induced cachexia. Muscle weight was measured at the end of the experiment, normalized to the body weight measured on the day cells were inoculated and then expressed as % change from the Sham group (non-tumor control group that received the solution without cells). Values are expressed as means ± SEM (n=10-11); *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.01$ versus non-tumor group by unpaired t-test comparison.

Figure 4: Calf Muscle Volume assessed by MRI. Calf muscle volume was measured in CT-26 induced cachexia with MRI under anesthesia with isoflurane, calculated with imaging software and represented as mm^3. All values are expressed as means ± SEM (n=10-11); *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.01$ versus non-tumor group by unpaired t-test comparison.

Figure 5: The isometric force of the gastrocnemius complex. A) CT-26 mouse colon cancer and **B)** A2058 human melanoma -induced cachexia. The sciatic nerve was stimulated using 1Hz electrical stimulation (Twitch) and increasing frequencies ranging from 10 to 160 Hz (Tetanus) with a stimulating electrode. The force of muscle contraction produced by the gastrocnemius-soleus-plantaris muscle complex in response to electrical stimulation of the sciatic nerve was

acquired and calculated with a data acquisition system. Force is represented in gram; values are expressed as means + SEM); *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.01$ versus non-tumor group by unpaired t-test comparison.

Table 1: List of established cachectic cell lines.

DISCUSSION:

Cancer patients with reduced skeletal muscle mass resulting in functional impairment are at an elevated risk for toxicities due to chemotherapy treatment and increased overall mortality. Here we demonstrate that a calf volume decrease is measurable by MRI at early stages of the disease progression (7 days post cell inoculation), whereas bodyweight decrease could only be observed about one week later (15 days post cell inoculation). Equally, it is reported that body weight loss alone was not able to reflect muscle fiber atrophy in cancer cachexia patients[16]. Therefore, calf volume could be used as an early, non-invasive marker for initiation of a therapeutic treatment. When choosing an experimental model for cancer cachexia it is important to consider as a first choice the cell lines that lead to the smallest tumor burden (**Table 1**). Indeed, the window to test therapeutic compounds will be less limiting.

Furthermore, we could observe that even with a marginal loss of body weight (loss of 5% in A2058 tumor-bearing animals), hind limb muscle mass and function were significantly impacted, as shown by the pronounced gastrocnemius complex atrophy and significantly reduced evoked force. This further highlights the importance of using muscle mass and functions, not only relying on bodyweight as the readout to assess muscle wasting conditions. The use of MRI could be expanded to evaluate comorbidities associated with cancer cachexia, such as cardiac alterations which are often observed in cancer cachexia[17, 18], although the access to MRI might be limited.

Importantly, hind limb muscle mass, volume and evoked force measurement could also be used to monitor the progression of cachexia and/or loss of muscle mass and functions, in other animal models such as anorexia-cachexia syndrome, cardiac or burn-induced cachexia, sarcopenia and disuse-induced muscle atrophy models. In particular, the measurement of clinically applicable readouts such as muscle volume by MRI non-invasively as well as muscle function allows more systemic assessment of the wasting condition, which gives a great advantage over the method described previously[19]. It would be more important in the future to take such an integrated approach to assess the effect of therapeutic interventions for the treatment of cachexia and muscle wasting conditions.

ACKNOWLEDGMENTS:

We thank the Muscle Diseases Group at Novartis Institutes for BioMedical Research (NIBR) for their enthusiastic support, along with the rest of the NIBR community. We particularly want to thank Michael Obrecht and Peter R Allegrini for their support.

DISCLOSURES:

Marie Jourdain, Stefan Melly, Serge Summermatter and Shinji Hatakeyama are employees of the Novartis Institutes of Biomedical Research.

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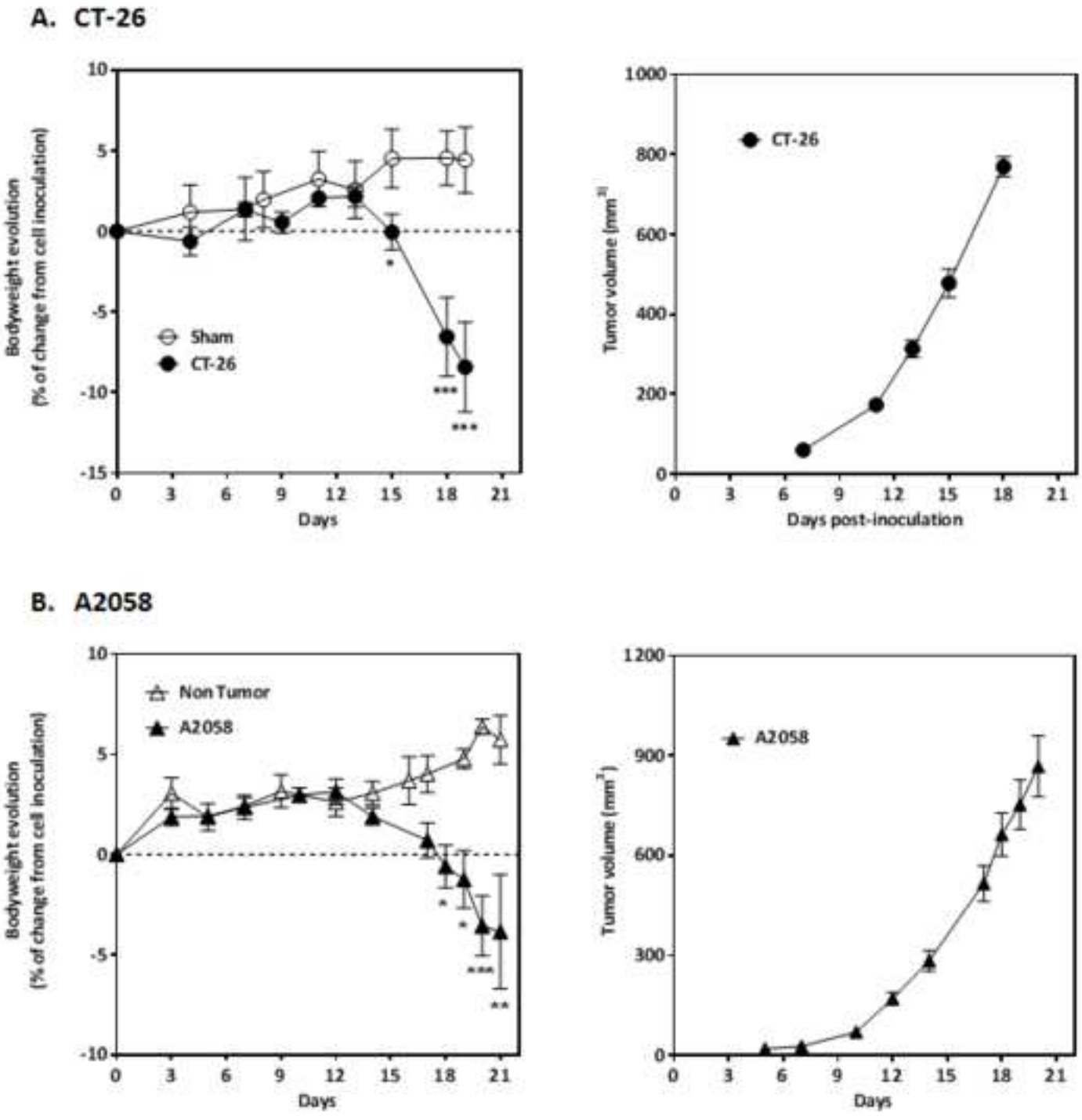

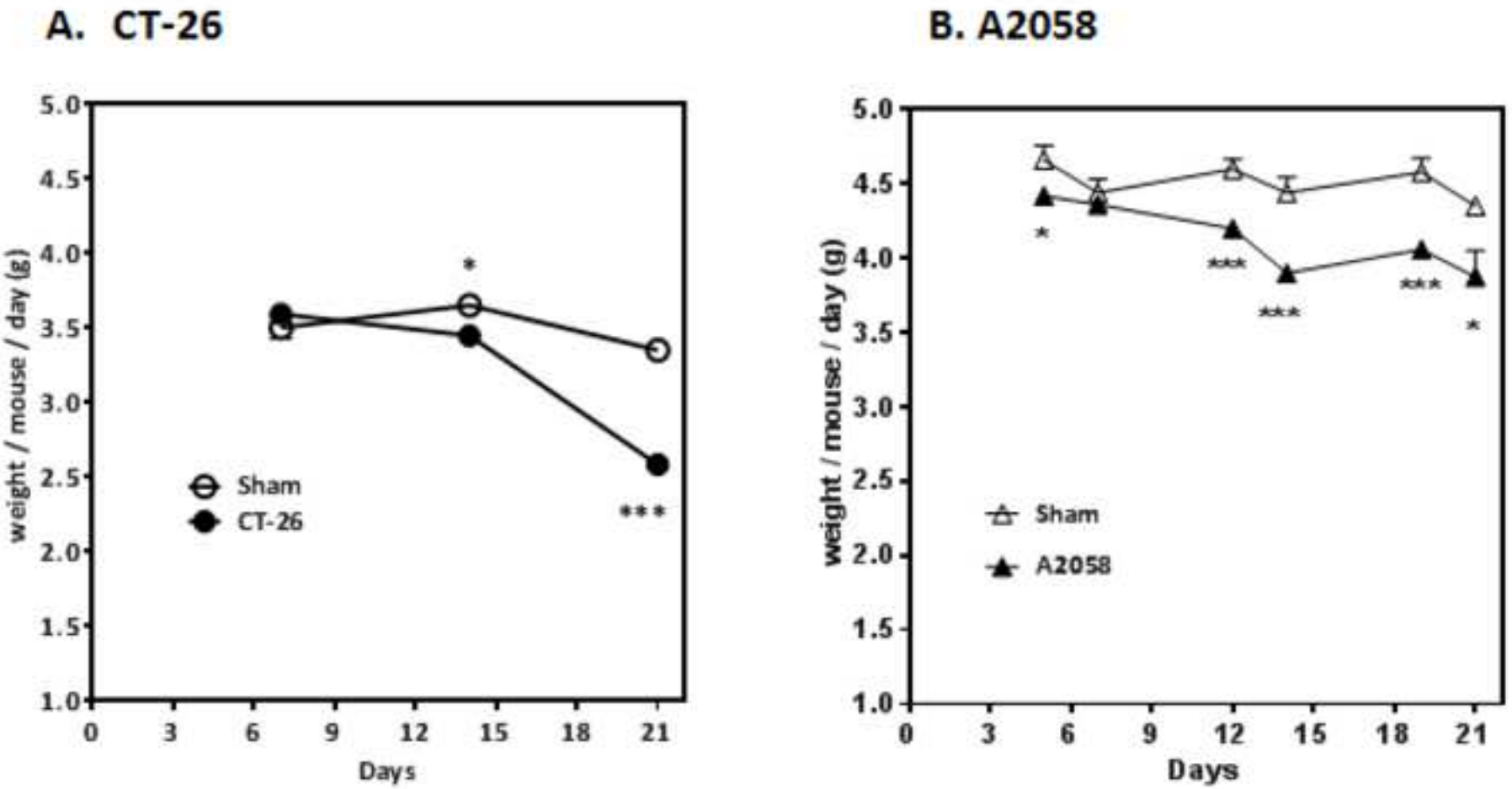

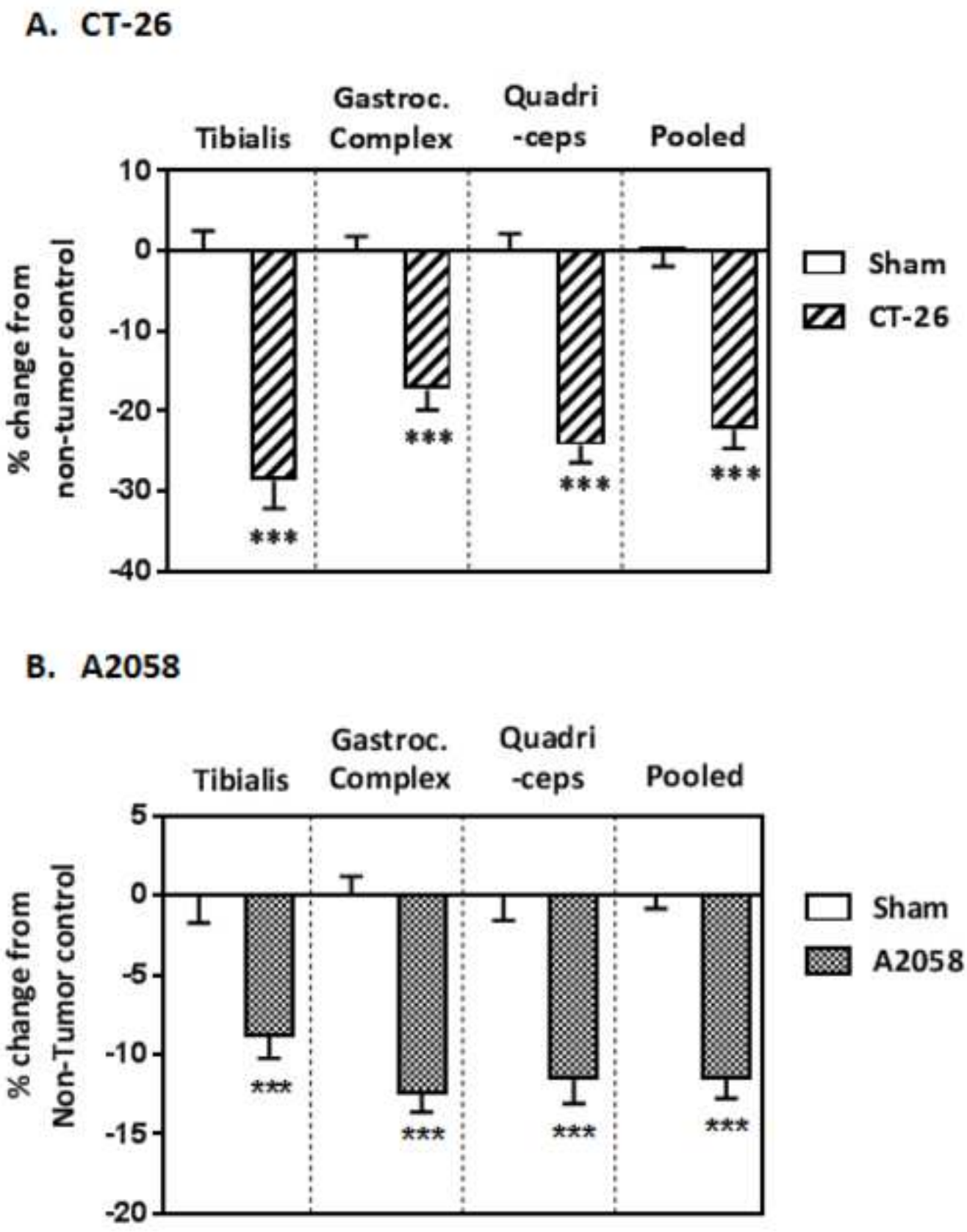

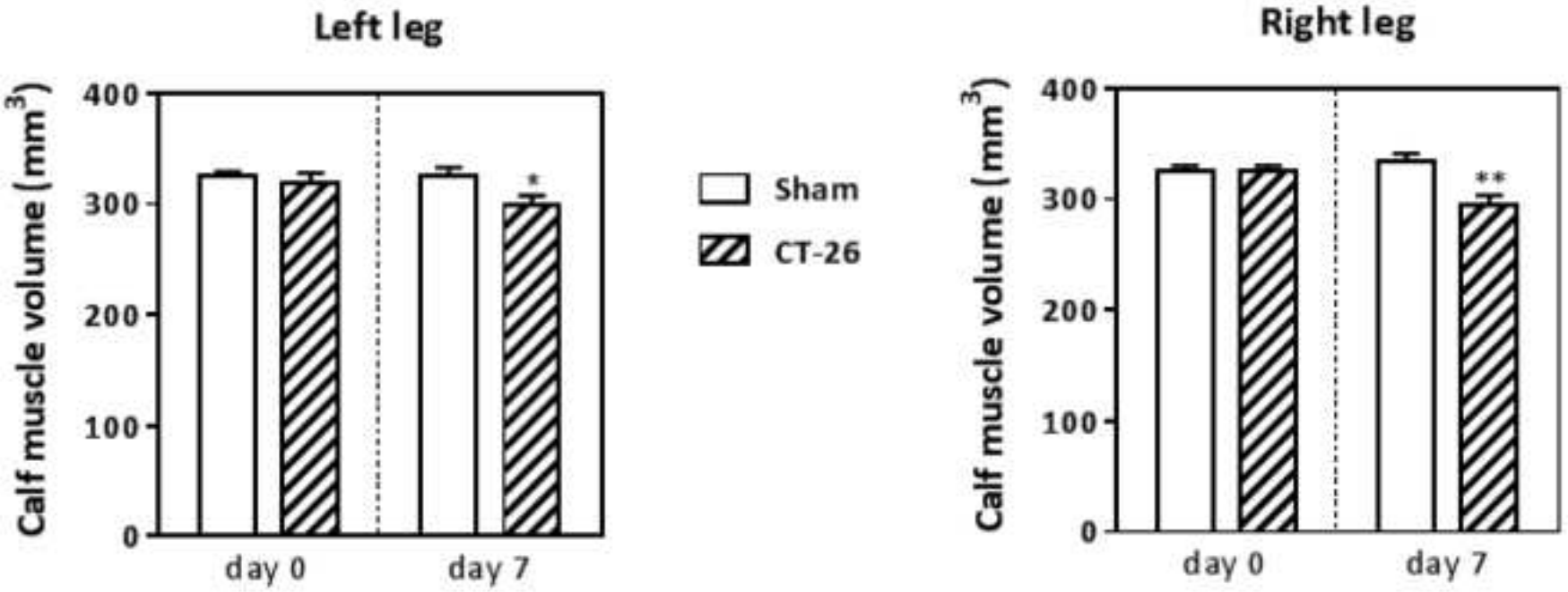

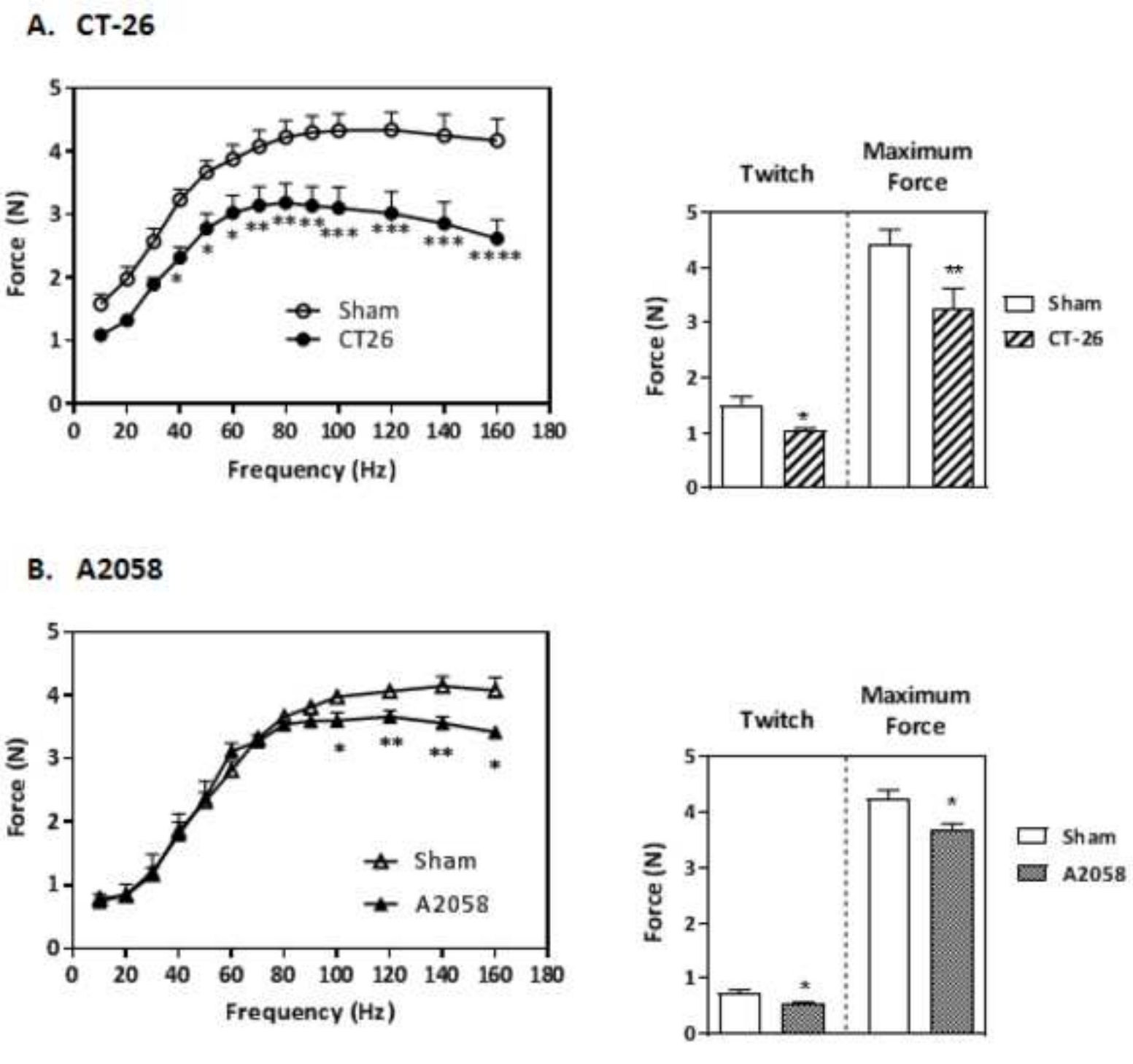

Cell Line	Origin	Host	Number to Inoculate	Days until termination
A2058	Skin Melanoma	Nude	$3\text{-}5 \times 10^6$	20-30
A375M	Skin Melanoma	Nude	5×10^6	21-32
C32	Skin Melanoma	Nude	3×10^6	31-34
CT-26	Colon Carcinoma	Balb/C	3×10^5	18 - 25
G-361	Skin Melanoma	Nude	8×10^5	27-42
LS-180	Colon Carcinoma	Nude	$3\text{-}5 \times 10^6$	16-22
MKN-1	Gastric Carcinoma	Nude	8×10^6	41-48
PC-3M	Prostate Carcinoma	Nude	3×10^6	20-35
TOV-21G	Ovary Carcinoma	Nude	$3\text{-}5 \times 10^6$	25-35

Reason for termination
Tumor size and body weight loss
Tumor size and body weight loss
Bodyweight loss
Tumor size and body weight loss
Body weight loss
Tumor size
Tumor size and body weight loss
Body weight loss
Body weight loss

Materials Table

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Name	Company
Force Transducer	Grass Instruments
Force Transducer	ADInstruments GmbH
LabChartPro	
Power Lab	
Stimulus Generator	
MRI Avance 7 T / 30 cm wide-bore instrument	Bruker BioSpin Inc
Matlab	Mathworks
physiology monitoring system	SA Instruments Inc
mouse colon cancer cell line CT-26	
human A2058 melanoma cell line	ATCC

Catalog Number	Comments
FT03	Not for sale in EU anymore
MLT1030/A	
PL3504/P	
FE180	
	obtained from Dr. Chatenay-Rivauday at Novartis Pharma AG (Basel, Swit:

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Author(s): Jourdain Marie, Melly Stefan, Summermatter Serge, Hatakeyama Shinji

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CORRESPONDING AUTHOR:

Name:	Hatakeyama, Shinji
Department:	MusculoSkeletal Diseases, Novartis Institutes for Biomedical Research
Institution:	Novartis Pharma AG
Article Title:	Mouse models of cancer-induced cachexia: hind limb muscle mass and evoked force as readouts
Signature:	 Date: 01 September 2017

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Reviewers' comments:

Reviewer #1: Manuscript Summary:

The manuscript by Jourdain and coworkers describe two mouse models of cancer cachexia, the first already well established and characterized by several laboratories (including PMID: 27929469 in this same journal) and the second reported in a single paper (PMC5435723). The manuscript highlights methodological aspects relevant for providing reliable investigational tools. The subject is relevant and deserves attention, however some points should be clarified and/or improved.

We would like to thank the reviewer for the constructive comments, which are highly appreciated. We have now modified the manuscript according to the reviewer's suggestions.

Major Concerns:

- Cancer cell lines: please provide a reference for the source (Cell bank, original paper where the line was established, commercial provider, etc.). Moreover, clarify whether CT26 cells are similar or the same cells that elsewhere are called C26.

The mouse colon cancer cell line CT-26 was obtained from Dr. Chatenay-Rivauday at Novartis Pharma AG (Basel, Switzerland) and originally provided by Dr. Isaiah J Fidler's lab at the MD Anderson Cancer Center in Houston TX. We now indicated the source of the cells and have also added a corresponding reference (Int J Cancer. 1992 Aug 19;52(1):98-104).

- From the text it is unclear whether final body weight change in comparison to the initial body weight is takes in consideration the tumor mass. Please provide both data (with and without tumor mass).

Tumor weight was not subtracted from final body weight when % of change from cell inoculation was calculated. This is now specified in the manuscript (legend Figure 1). In the case of CT-26, it was -8.4% with tumor weight and -11% without tumor (i.e. after subtracting tumor weight).

- Please explain the reason for choosing the adopted temporal endpoint. One might argue that also A2058 cell induce severe cachexia if allowed to growth for longer.

We fully agree with the reviewer and would like to thank for the comment. The endpoints have been selected based on cantonal regulations for animal experimentation. The experimental license BS2186 granted by the cantonal authorities of Basel requires animals to be euthanized as soon as certain predefined cut-off criteria are reached. In case of experiments involving CT-26 cells, mice reached the bodyweight loss cut-off (-20%) faster than the tumor volume cut-off (1500 mm^3). In case of experiments involving A2058 cells, mice reached the tumor volume cut-off (1500 mm^3) earlier and had to be sacrificed despite moderate bodyweight loss. This is now specified in the manuscript.

- As for the food intake, few points are present in the curves in comparison to the body weight data. I suppose that each point represent the average amount of food intake consumed in the period starting from the previous measurement. Such approx. impedes to highlight a potential severe anorexia present in the last days before sacrifice.

Food intake is shown as the average food consumption during the indicated days. We now more clearly state this in the manuscript.

- Please comment on the value and feasibility of measuring evoked force as compared to voluntary strength (e.g. grasping test or others). Moreover, the force in the graph is expressed in grams, not the right measure unit (Newton), please convert.

The disadvantage of other tests (e.g., grasping test) is that they depend on the motivation of the animals, which is an enormous confounding factor. The evoked force occurs under anesthesia and is triggered by increasing electrical stimulation, which allows assessments over the entire range of muscle contractions from the minimal to the maximal force. We have now added a corresponding explanation. The unit has been converted to Newton.

- MRI for detecting calf volume reduction was performed only at 7 days of tumor growth. Such result is intriguing since highlights the precocious loss of muscle mass, however it would be useful to provide a time course or at least the final point before sacrifice in order to validate the data as compared to muscle mass measured at necropsy.

We fully agree with the reviewer. We wanted to assess, whether at the 7, which is prior to body weight loss, there is already a muscle weight loss which could be used as an early, non-invasive marker for muscle wasting. We now highlight our rationale in the manuscript.

Minor Concerns:

- Please clarify better the area where cell injection is performed.

Cells are injected subcutaneous on the left or right upper flank side, in order to allow proper grabbing for tumor measurements or other manipulation without touching the tumors. This is now described in the manuscript.

Reviewer #2: Manuscript Summary:

The authours address an important question in muscle wasting field: can skeletal muscle wasting precede body weight loss in cancer bearing-mice? Two unrelated cancer cachexia models are used the C26-bearing mouse and the human melanoma bearing-ones. The first is in an immunocopetent backgroud and the second in immunodeficient mice.

The comparison between the two models helped to answer the main question of the paper

Major Concerns:

Some important dataset are missing:

In fig 1 survival plot shall be added

We are not allowed to run survival experiments under our experimental licenses. However, we have recently published surrogate survival plots based on our predefined cut-off criteria (body weight loss or tumor size), which are now specified in the method and have now added the corresponding reference.

In Fig 2 is food intake measured for each animal that has been kept alone in each cage? Or is the food intake the mean of the food eaten from multiple animals for the same cage and divided for the n? That should be clarified

The experimental license BS2186 granted by Basel-City cantonal authorities does not allow us to keep animals alone unless there is a major incompatibility. Therefore the food intake data is calculated by dividing the total amount of food eaten per cage by the number of mice present in this cage. This is now specified in the manuscript.

In fig 3 soleus decrease in size shall be shown alone as example of oxidative muscle, please show that out. At which day from tumor injection have these muscles been weighed? If different for each mouse (data come from a survival experiment), they cannot plot altogether and the experiment has to be repeated choosing a predefined timepoint. The same holds for the fig 3B

In these experiments, soleus muscle weight is not available because we measured the weight of gastrocnemius-plantaris-soleus muscle complex and did not separate each muscle. With regard to the timing to weigh the muscles, it was performed one to two days after the last measurements of body weight and tumor volume, not different for each mouse.

in fig 4 what are the calf measurements by MRI in A2058-carrying mice? Please add them, if they do not show muscle wasting ,discuss why that in the appropriate section

MRI measurement was not performed with A2058.

Minor Concerns:

CT-26 shall be replaced with C26

why just 3X105 cells of C26 are injected if most researchers do 1 million or half millions of cells?

The starting cell number has been determined in pilot experiments. The amount indicated was appropriate to induce substantial tumor growth with sufficient window for later treatment in the model. This is now more clearly stated in the manuscript.

cubic mm is unappropriate, please correct it as well as many mispellings and gramatcal mistakes throughout all the text

Cubic mm has been replaced with mm^3, and typos have been corrected.

Discussion shall be enriched with references and points coming out from the data provided

We thank for the suggestion. Discussion is enriched with references and key points from the data.

Editorial comments:

Changes to be made by the Author(s):

The changes requested by the editor have been applied and the essential steps of the protocol are highlighted in yellow.

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9. Please revise the Introduction to include all of the following:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

10. Please use S.I. abbreviation throughout the protocol.

11. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

12. 1.1.1-1.2.1: Please explain where is the cell line from? How do you put the frozen cell in culture? Do you thaw the cells or just transfer the cells from the cryovial to the tube and then add medium to it? How do you ensure the cell number at this stage?

13. 1.1.2.- 1.2.2: How do you passage the cells? Please add a note stating why 2-3 or 3-4 passages of the cells. What is the cell confluency while trypsinisation? What other criteria do you look for before ensuring cells are ready for further processing- cell count, etc?

14. 1.3.1 – How much cells are injected? Cell number/kg body weight? Do you check anything before and after cell injection? What is the age and gender of the mice being inoculated? Anything specific to keep in mind.

15. 2.1- This should come before the animal injection occurs. This will be the order of filming.

16. 3.2: Please provide GUI if this step needs to be filmed. The video will show how to do the things.

17. 4.2: Please explain do you use sterile instrument? How do you identify the Achilles tendon?

18. 4.4: Please explain when was the sciatic nerve exposed?

19. Line 178-181: Please mark as a note.

20. Please ensure that a single line space is left between each step and sub-step of the protocol. The font used us Calibri and font size is 12 with one inch margin on all sides.

21. Please highlight 2.75 pages or less (hard cut limit) of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

22. Please also ensure that the steps involving anesthesia, steps involving calculations and computational steps without GUI cannot be filmed.

23. Line 190: Please explain what is non- tumor group. What was injected in the non-tumor group? What is being measured here?

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c) Any limitations of the technique

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