**TITLE:**

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

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**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 rate1, 2. It is proposed that skeletal muscle loss is the most prominent event in cancer cachexia and cannot be fully reversed by conventional nutritional support3-5. 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 syngeneic8-10, and one xenograft9. Furthermore, readouts such as Magnetic Resonance Imaging (MRI)12 and evoked force measurement13, 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 mm3. 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-2615 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% CO2.

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 106 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% CO2.

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 106 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 O2 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 107 cells in case of CT-26 or 3-5 x 107 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 × width2)/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 CO2 in the following cases: when body weight loss is close to 20%, tumor volume reaches 1,500 mm3, 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 CO2, 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 (mm3).

**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 O2 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 CO2 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 mm3 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 mm3. 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 × width2)/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 mm3. 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 patients16. 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 cachexia17, 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 previously19. 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.

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**DISCLOSURES:**

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

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