The animal cachexia score (ACASCO)

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Abstract

Background: None of the published studies involving cancer cachexia experimental models have included a measure of the severity of the syndrome like the scoring system previously developed for human subjects. The aim of the present investigation was to define and validate a cachexia score usable in both rat and mouse tumor models.

Methods: In order to achieve this goal, we included in the study one rat model (Yoshida AH-130 ascites hepatoma) and two mouse models (Lewis lung carcinoma and Colon26 carcinoma). The Animal cachexia score (ACASCO) includes five components: (a) body and muscle weight loss, (b) inflammation and metabolic disturbances, (c) physical performance, (d) anorexia, and (e) quality of life measured using discomfort symptoms and behavioral tests.

Results: Using the ACASCO values, three cut-off values were estimated by applying hierarchical cluster analysis. Four groups were originally described, one exactly below the observed mean, a second exactly over the mean, and two other groups adjusted to every cue (inferior and superior). The three cut-off values were estimated through maximization of the classification function. This was accomplished by using a similarity matrix based on the metric properties of the variables and assuming multinormal distribution. The results show that the four groups were: no cachexia, mild cachexia, moderate cachexia and advanced cachexia.

Conclusions: The results obtained allow us to conclude that the score could be very useful as an endpoint in pre-clinical studies involving therapeutic strategies for cancer cachexia. The potential usefulness of ACASCO relates to the primary endpoint in pre-clinical cancer cachexia drug evaluations.

Keywords
anorexia, cachexia, classification, physical performance, quality of life, score, wasting, weight loss
Cancer cachexia is a multi-organic syndrome that affects the majority of cancer patients. It is characterized by both anorexia and metabolic abnormalities that lead to weight loss and, particularly, muscle wasting. It becomes essential, in order to treat cachexia adequately, to assess the cancer patient according to the degree of his/her cachexia. It is for this reason that our research group introduced the so-called CASCO (Cachexia Score),\(^1\) for assessing cancer patients. CASCO was recently validated.\(^2\) None of the published studies involving cancer cachexia experimental models include a measure of the severity of the syndrome like the previously reported CASCO system for human subjects.\(^1,2\) Therefore, the aim of the present investigation was to define and validate a cachexia score usable in both rats and mice. In order to achieve this goal, the study included one cachexia rat model (Yoshida AH-130 ascites hepatoma) and two mouse models (Lewis lung carcinoma and Colon26 carcinoma).

The score for classifying the degree of cachexia in experimental animals was termed the Animal Cachexia Score (ACASCO) and the components of this scoring system are the following: (a) body and muscle weight loss, (b) inflammation and metabolic disturbances, (c) physical performance, (d) anorexia, and (e) quality of life measured using discomfort symptoms and behavioral tests. The components and the details of the measured parameters can be seen in Table 1 and they are based on the components used for the classification of cancer cachexia in human patients.\(^1,2\)

Briefly, body weight loss is an essential component of all definitions of cachexia.\(^3\) Moreover, muscle wasting is the main phenomenon associated with body weight loss. It is therefore important to assess any changes that may occur in muscle mass. The second component of ACASCO includes inflammation and metabolic disturbances. Inflammation is a very important component of the cachetic response.\(^4,5\) In addition to inflammation, there are a number of metabolic disturbances that are present in most cachectic animals, such as glucose intolerance, anemia and low levels of plasmatic albumin, among others, most of them included in ACASCO (see Table 1). The third component relates to physical performance. Even if there is a relatively small decrease in muscle mass due to the cachetic syndrome, there may be a significant decrease in physical activities related to muscle performance.\(^6,7\) Anorexia (ANO) constitutes the fourth parameter included in ACASCO. Anorexia is an important component of cachexia in many types of diseases.\(^8,9\) A decrease in food intake, by itself, promotes changes in quality of life and also conditions many metabolic alterations. Finally, the last component of CASCO is quality of life (QoL). Quality of life reflects not just changes in weight and physical performance but also metabolic alterations.\(^5,7\) It is therefore essential to take it into consideration in the scoring system.

The five different factors mentioned clearly interact with each other and represent the most important set of variables that indicate the severity of the cachectic syndrome. Bearing all this in mind, the results obtained should allow for not only a qualitative but also a quantitative classification of the cachectic syndrome in experimental animals. ACASCO may then be related to different types of treatments according to the severity of cachexia.

2 | METHODS

2.1 | Experimental tumor models

Five-week-old male Wistar rats (for the AH-130 tumor model), C57BL/6 mice (for LLC tumour model) or Balb/C mice (for C26 tumour model) weighing about 20 g (all from Harlan, Barcelona, Spain) were housed in individual cages and maintained at a constant temperature of 22 ± 2°C with a regular dark-light cycle (light from 08:00 am to 20:00 pm), with free access to food and water during the whole experimental period. All animal manipulations were made in accordance with the European Community guidelines for the use of laboratory animals. They were cared for in compliance with the Policy on Humane Care and Use of Laboratory Animals (ILAR 2011) and in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The animals were kept in individual cages for a week prior to the beginning of the experimental protocol (adaptation period). No hyperphagia was observed during this period. All the animals were alive at the end of the experiments. On the day of sacrifice, the animals were weighed and anesthetized with an ip injection of ketamine/xylazine mixture (3:1) (Imalgene® and Rompun® respectively). Tissues were rapidly excised, weighed, and frozen in liquid nitrogen.

2.1.1 | AH-130 Yoshida ascites hepatoma

Wistar rats were randomized and divided into two groups, namely controls (C) and tumor bearers (TB). Experimental cachexia was obtained through ip injection of \(10^8\) AH-130 Yoshida ascites hepatoma cells, obtained from exponential tumours, as described previously,\(^10\) into male Wistar rats. On days 2, 4, 6, 8, 10 and 11 after tumor transplantation, the animals were sacrificed and the blood and tissues were rapidly excised for further analysis. Five control rats and six tumor-bearing rats were sacrificed in each time point.

2.1.2 | Lewis lung carcinoma

C57BL/6 mice were randomized and divided into two groups, namely controls (C) and tumor bearers (TB). Mice received an intramuscular (hind leg) inoculum of \(5 \times 10^5\) Lewis lung carcinoma cells obtained from exponential tumors. The Lewis lung carcinoma is a highly cachectic, rapidly growing mouse tumor containing poorly differentiated cells, with a relatively short doubling time.\(^11\) On days 4, 6, 10, 14, 18 and 20 after tumor transplantation, the animals were sacrificed and blood and tissues were rapidly excised for further analysis. Five control mice and six tumor-bearing mice were sacrificed at each time point.

2.1.3 | Colon26 carcinoma

Balb/C mice were randomized and divided into two groups, namely controls (C) and tumor bearers (TB). Tumor-bearing mice
were inoculated subcutaneously in the back with $5 \times 10^5$ Colon26 carcinoma cells. Colon26 cells were maintained in vitro in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL sodium pyruvate, 2 mmol/L L-glutamine, at 37°C in a humidified atmosphere of 5% CO₂ in air. The day of tumor implantation the cells were trypsinized, re-suspended in sterile saline and subsequently implanted in the back of the animals at the concentration indicated above. Mice were sacrificed under anesthesia at days 6, 8, 14, 15, 20 and 24 after tumor implantation. Five control mice and six tumor-bearing mice were sacrificed at each time point.

| TABLE 1  | ACASCO components and their relative scores |
|----------|--------------------------------------------|
| Symptom  | %  | Measurement | Total score | Parameter | Criteria | Points |
| Body and GSN weight loss | 40 | Body weight loss (BWL) | 32 | Rat | <5% | 0 |
|  |  |  |  |  | ≥5% | 8 |
|  |  |  |  |  | ≥15% | 16 |
|  |  |  |  |  | ≥30% | 24 |
|  |  |  |  |  | ≥50% | 32 |
| GSN weight loss | 8 |  |  | No change in muscle weight or changes <10% | BWL points | x 0 |
|  |  |  |  | Change >10% | BWL points | x 1 |
|  |  |  |  | Change >20% | BWL points | x 1.25 |
| Inflammation/metabolic disturbances | 30 |  |  | Difference vs control | 8 |
|  |  |  |  |  | 8 |
|  |  |  |  |  | 3.5 |
|  |  |  |  |  | 3.5 |
|  |  |  |  |  | 3.5 |
| Physical activity | 10 |  | 6 | Total physical activity | 0 to 9.99 | 0 |
|  |  |  |  |  | 10 to 20.99 | 3 |
|  |  |  |  |  | ≥21 | 6 |
|  |  |  |  |  | 0 to 4.99 | 0 |
|  |  |  |  |  | 5 to 20.99 | 1 |
|  |  |  |  |  | 21 to 30.99 | 2 |
|  |  |  |  |  | ≥31 | 4 |
| Anorexia | 10 |  | 10 | Decrease of food intake | 0 to 4.99% | 0 |
|  |  |  |  |  | 5% to 19.99% | 5 |
|  |  |  |  |  | 20% to 29.99% | 7 |
|  |  |  |  |  | ≥30% | 10 |
| Quality of life | 10 |  | 1 | Discomfort symptoms | 0 - 2 | 0 |
|  |  |  |  |  | 3 - 6 | 0.5 |
|  |  |  |  |  | 4 - 6 | 1 |
|  |  |  |  |  | 0 to 0.99% | 0 |
|  |  |  |  |  | 10% to 30.99% | 1 |
|  |  |  |  |  | 31% to 60.99% | 2 |
|  |  |  |  |  | ≥61% | 4 |
| Intruder-resident paradigm | 4 |  | 5 | Forced swim test | 0 to 9.99% | 0 |
|  |  |  |  |  | 10% to 30.99% | 2 |
|  |  |  |  |  | 31% to 60.99% | 3 |
|  |  |  |  |  | ≥61% | 5 |
2.2 | ACASCO measurements

All the measurements are expressed as a percentage of the difference between non-tumor-bearing animals and tumor-bearing animals, at the time of sacrifice (see Experimental tumor models section).

2.2.1 | Body and muscle weight loss

Body weight was calculated by subtracting the tumor weight from the weight of the tumor-bearing (TB) animal at the moment of sacrifice, and the weight loss was expressed as a percentage of the weight of the control group at sacrifice. The criteria used for scoring are depicted in Table 1 and are different for rat and mouse models. Muscle weight loss was measured as the percentage of gastrocnemius (GSN) muscle weight loss corrected for initial body weight and referenced to the control group (GSN loss = GSN weight of TB group at sacrifice/GSN weight of control group at sacrifice) × 100.

This formula takes into consideration the variation in muscle weight related to the non-tumor bearing control group. Gastrocnemius muscle was chosen because it is one of the most studied muscles in the experimental cachexia field and its weight reflects cachexia severity.13-15 In the case of the rat model, the scoring criteria for body weight loss (BWL) is described in Table 1: <5%, 0 points; ≥5%, 8 points; ≥15%, 16 points; ≥30%, 24 points; ≥50%, 32 points. For the mouse models the scoring criteria for body weight loss were: <2%, 0 points; ≥2%, 8 points; ≥12%, 16 points; ≥15%, 24 points; ≥25%, 32 points (Table 1). GSN weight loss scoring criteria are also depicted in Table 1: <10%, BWL score × 0; >10%, BWL score × 0; >20%, BWL score × 1. Despite the severe losses seen, fat wasting was not included in the cachexia score because (a) it was not considered in the original CASCO scoring system used in humans, and (b) loss of muscle is considered the main component of cancer cachexia, rather than loss of fat.

2.2.2 | Inflammation and metabolic disturbances

The scoring for inflammation and metabolic disturbances indicated in Table 1 is based on the existence of statistically significant differences in values between the TB and control values. Depending on the metabolite/inflammatory marker the punctuation is different (Table 1).

ELISA

IL-6 (pg/mL) and SAA (ng/mL) plasma levels were detected using commercially available mouse and rat ELISA kits, according to the manufacturer instructions: mouse IL6 and rat IL-6 kit (Diaclone SAS, France); mouse SAA kit (Abnova, Taiwan); and rat SAA kit (Cusabio Wuhan, China).

Metabolic disturbances

Plasma albumin (g/dL), triacylglycerides (mg/dL) and glucose (mg/dL) were analyzed using a METROLAB 2300 autoanalyzer, based on analysis of colorimetric reactions. Hematocrit

Total blood was withdrawn from anesthetized mice by cardiac puncture and collected in heparinized tubes. A drop was used to fill hematocrit capillary tubes, which were centrifuged in a hematocrit centrifuge for 5 minutes at 800g. Hematocrit was calculated as the volume percentage of packed red cell volume in total blood.

2.2.3 | Physical performance

Total physical activity

Total physical activity (IR ACTIMETER System and ACTITRAK software from PANLAB, Barcelona) was determined during the last 24 hours before the sacrifice of the animals in control and tumor-bearing animals, using activity sensors that translate individual changes in the infrared pattern, caused by movements of the animals, into arbitrary activity counts. For the measurements, animals remained in their home cage. A frame containing an infrared beam system was placed on the outside of the cage; this minimized stress to the animals.16 The scoring of total physical activity (TPA) is indicated in Table 1. The parameter is referenced to the control group (TPA = TPA of TB group at sacrifice/mean TPA of the control group at sacrifice) × 100. A decrease in TPA of <9.99% in relation to the control group scores 0 points, while a decrease from 10 to 20.99 scores 3 points, and a decrease over 21% scores 6 points (Table 1).

Grip force assessment

Skeletal muscle strength (handgrip strength) in the experimental animals was quantified by the grip strength test.17,18 The grip-strength device (Panlab-Harvard Apparatus, Spain) comprised a pull bar connected to an isometric force transducer (dynamometer). Basically, the grip strength meter was positioned horizontally and the rats and mouse were held by the tail and lowered towards the device. The animals were allowed to grasp the pull bar and were then pulled backward in the horizontal plane. The force applied to the bar just before the animal lost grip was recorded as the peak tension. At least three measurements were taken per rat on both baseline and test days, and the results were averaged for analysis. Grip force (GF) was measured in g/g initial body weight.16 The scoring of grip force is indicated in Table 1. The parameter is referenced to the control group (GF = GF of TB group at sacrifice/mean GF of the control group at sacrifice) × 100. A decrease in GF of <4.99% in relation with the control group scores 0 points, from 5 to 20.99 scores 1 point, from 21% to 30.99% scores 2 points and over 31% scores 4 points (Table 1).

2.2.4 | Anorexia

Anorexia was assessed by measuring total food intake (TFI). The scoring is indicated in Table 1. The parameter is referenced to the control group (TFI = FI of TB group at sacrifice/TFI of the control group at sacrifice) × 100. A decrease in TFI of <4.99% in relation with the control group scores 0 points, from 5 to 19.99 scores 5 points, from 20% to 29.99% scores 7 points and over 30% scores 10 points (Table 1).
2.2.5 | Quality of life

In order to estimate the quality of life of the animals, several tests were performed.

Discomfort symptoms

The following symptoms were monitored: (a) piloerection, (b) diarrhea or constipation, (c) hunched posture, (d) tremors, (e) closed eyes and (f) red tears (chromodacryorrhea). Points to establish the score of discomfort symptoms are described in Table 1: 0-2 symptoms, 0 points; 3-6 symptoms, 0.5 points; 4-6 symptoms, 1 point.

Intruder-resident paradigm

This paradigm is based on the establishment of a territory by a male and its defense against unfamiliar male intruders. It consists of introducing a male, the intruder, into the home cage of another male, the resident, with the former being defeated by the latter.19 In the cachexia rat model, the interaction time between the intruder and the resident was measured during a 2-minute interval. The scoring was as follows: a decrease of the interaction compared with the control group of 0-9.99%, 0 points; 10%-30%, 1 point; 31%-60%, 2 points; 61%-100%, 4 points (Table 1). In the cachexia mouse models, resident-intruder interaction was evaluated based on rating the level of interest of the resident in the intruder:

| Parameter                      | Day 2 | Day 4 | Day 6 | Day 8 | Day 10 | Day 11 |
|-------------------------------|-------|-------|-------|-------|--------|--------|
| Body weight loss              | .093  | .085  | <.001 | <.001 | <.001  | <.001  |
| GSN weight loss               | .087  | .238  | .015  | <.001 | <.001  | <.001  |
| Serum amyloid A               | .015  | <.001 | <.01  | .025  | <.001  | <.001  |
| Interleukin-6                 | <.001 | <.001 | <.001 | <.002 | .007   |        |
| Albumin                       | .195  | .013  | .005  | .489  | .153   | .006   |
| Triglycerides                 | .012  | .004  | <.001 | .010  | .013   | .172   |
| Glucose                       | .260  | <.001 | <.019 | <.001 | <.005  | <.001  |
| Hematocrit                    | .011  | .022  | .408  | .017  | .002   | <.001  |
| Decrease in total physical activity | .032  | .020  | .0090 | .003  | <.001  | <.001  |
| Decrease in handgrip strength | .460  | .177  | .006  | .004  | .009   | <.001  |
| Decrease in food intake       | .500  | .406  | .007  | .002  | .010   | .002   |
| Discomfort symptoms           | <.001 | <.001 | <.001 | <.001 | <.001  | <.001  |
| Decrease in intruder-resident paradigm | .046  | <.001 | <.001 | <.001 | .002   | <.001  |
| Decrease in forced swim test  | .038  | .002  | .060  | .012  | <.001  | <.001  |

The results represent statistical differences observed between the mean values of the different groups. Body weight loss and gastrocnemius (GSN) weight loss calculations are described in the Methods section. Serum amyloid A (SAA) was expressed in ng/mL, IL-6 was expressed in pg/mL, albumin was expressed in g/dL, Triglycerides were expressed in mg/dL, glucose was expressed in mg/dL. Hematocrit was calculated as percentage of packed cell volume to total blood volume. Total physical activity (TPA) was referenced to the control group (TPA = TPA of TB group at sacrifice/mean TPA of the control group at sacrifice) × 100. Handgrip strength or grip force (GF) was measured in g/g initial body weight and the parameter was referenced to the control group (GF = GF of TB group at sacrifice/mean GF of the control group at sacrifice) × 100. Food intake (TFI) was referenced to the control group (TFI = FI of TB group at sacrifice/TFI of the control group at sacrifice) × 100. Discomfort symptoms are described in Table 1. The intruder-resident paradigm and forced swim test were evaluated using a rating scale established to determine the level of interest of the resident-intruder and the time during which the animals were trying to escape from the water, respectively (Table 1).

Forced swim test

A container filled with tap water at 26°C to a depth of 30 cm was used to carry out the forced swim test.20 Animal activity was registered with a video system for 90 seconds. The time during which the animals were trying to escape from the water was registered. After the test, the rats were dried and returned to their cages. The scoring was as follows: a decrease in relation to the control group of 0-9.99%, 0 points; 10%-30%, 2 points; 31%-60%, 3 points; 61%-100%, 5 points.

2.3 | Statistical analysis

Statistical analysis of the differences between the control and tumor-bearing animals at each time point was performed by means of the Student’s t test, with a significance level of α = 0.05, and correcting for reduction of type I error using the Bonferroni correction. Cluster analysis between groups was performed to determine the breakpoints within the scale and estimate the maximum inertia centroids values. The computer program IBM SPSS Statistic 20 was used to take account of data lost either experimental errors or problems related with the experimental animals.
| Parameter                          | Day 4  | Day 6  | Day 10 | Day 14 | Day 18 | Day 20 |
|-----------------------------------|--------|--------|--------|--------|--------|--------|
| Body weight loss                  | .431   | .348   | <.001  | <.001  | <.001  | <.001  |
| GSN weight loss                   | .103   | .203   | <.001  | <.001  | <.001  | <.001  |
| Serum amyloid A                   | .012   | .020   | <.001  | <.001  | <.001  | <.001  |
| Interleukin-6                     | .288   | <.001  | <.001  | .003   | .069   | .213   |
| Albumin                           | <.001  | <.001  | <.001  | <.001  | <.001  | <.001  |
| Triglycerides                     | <.001  | <.001  | <.001  | .128   | .399   | .028   |
| Glucose                           | .013   |        | .064   | .197   | .089   | .316   |
| Hematocrit                        | .394   | .007   | <.001  | <.001  | <.001  | <.001  |
| Decrease in total physical activity| .319   | .188   | <.001  | <.001  | .004   | <.001  |
| Decrease in handgrip strength     | .373   | .041   | <.001  | .018   | .133   | .004   |
| Decrease in food intake           | <.001  | <.001  | <.001  | <.001  | <.001  | <.001  |
| Discomfort symptoms               | <.001  | <.001  | <.001  | <.001  | <.001  | <.001  |
| Decrease in intruder-resident paradigm| .0042  | .427   | .002   | <.001  | <.001  | <.001  |
| Decrease in forced swim test      | .090   | .410   | .011   | <.001  | <.001  | <.001  |

The results represent statistical differences observed between the mean values of the different groups. Body weight loss and gastrocnemius (GSN) weight loss calculations are described in the Methods section. Serum amyloid A (SAA) was expressed in ng/mL, IL-6 was expressed in pg/mL, albumin was expressed in g/dL, Triglycerides were expressed in mg/dL, glucose was expressed in mg/dL. Hematocrit was calculated as percentage of packed cell volume to total blood volume. Total physical activity (TPA) was referenced to the control group (TPA = TPA of TB group at sacrifice/mean TPA of the control group at sacrifice) × 100). Handgrip strength or grip force (GF) was measured in g/g initial body weight and the parameter was referenced to the control group (GF = GF of TB group at sacrifice/mean GF of the control group at sacrifice) × 100). Food intake (TFI) was referenced to the control group (TFI = TFI of TB group at sacrifice/TFI of the control group at sacrifice) × 100). Discomfort symptoms are described in Table 1. The intruder-resident paradigm and forced swim test were evaluated using a rating scale established to determine the level of interest of the resident-intruder and the time during which the animals were trying to escape from the water, respectively (Table 1).

3 | RESULTS

Table 1 depicts the different components of the Animal Cachexia SCORE (ACASCO) system. As can be seen, the body and muscle weight loss score contributes most to the overall score (40%), followed by inflammation and metabolic disturbances (30%) and then, with the same weighting, physical performance (10%), anorexia (10%) and quality of life (10%).

The different criteria used to quantify these components are also included in Table 1, together with the total score of each component. It can be seen that there are some differences in the scoring system between rats and mice. These include the scoring criteria for weight loss and quality of life (specifically the intruder-resident paradigm). Comparing ACASCO and CASCO, the measurements of quality of life for experimental animals were based on behavioral tests instead of the typical questionnaires used in humans. Another difference between ACASCO and CASCO relates to one of the measurements of inflammation; serum amyloid A (SAA) used here is the main acute phase protein in rodents while C-reactive protein (CRP) is the equivalent for humans. The rest of the measurements are basically the same between ACASCO and CASCO.

In order to follow the differences in these parameters in the different experimental models used, we have included the data in Tables 2-4 for the rat model (Yoshida AH-130 ascites hepatoma) and the two mouse models (Lewis lung carcinoma [LLC] and Colon26 carcinoma [C26]), respectively. Since the tumor models used in this study have different growth rates and therefore survival is different, we chose different disease progression time points (days of sacrifice) in each model, which were adapted to the growth characteristics for each cancer. The data in Tables 2-4 show the changes in the parameters analyzed in relation to the period of tumor growth. The numbers represent the statistical significance (P value) vs non-tumor bearing animals. Among the most relevant data, it can be seen that body weight loss affected the Yoshida AH-130 ascites hepatoma model from day 6 onwards (Table 2), the Lewis lung carcinoma model from day 10 onwards (Table 3) and the Colon26 carcinoma from day 6 onwards (Table 4).

Inflammation, measured using SAA as a marker, was present in the Yoshida AH-130 and LLC models from the first day of observation and from day 8 in the C26 model. Similar results were obtained with interleukin-6 (IL-6). It must be emphasized that weight loss and inflammatory response are considered the main components of the cachectic response.

Interestingly, the decrease in physical activity (this component reflects changes in muscle performance) took place from day 6 in all the tumor models considered (Tables 2-4); decrease in total physical activity and decrease in handgrip force. Many other measurements were also significantly altered at the early tumor stages (anorexia and quality of life components).

Using the ACASCO values, three cut-off values were estimated by applying hierarchical cluster analysis. Four groups were originally
TABLE 4 Colon 26 carcinoma model: statistical analysis of the differences between the changes observed vs the control (non-tumor bearing) animals

| Parameter                        | Day 6  | Day 8  | Day 14 | Day 15 | Day 20 | Day 24 |
|----------------------------------|--------|--------|--------|--------|--------|--------|
| Body weight loss                 | <.001  | .076   | .014   | .011   | <.001  | <.001  |
| GSN weight loss                  | .246   | .033   | .017   | .020   | <.001  | <.001  |
| Serum amyloid A                  | .122   | .002   | <.001  | <.001  | <.001  | <.001  |
| Interleukin-6                    | .083   | .228   | <.001  | <.001  | <.001  | <.001  |
| Albumin                          | .005   | .044   | .002   | .003   | <.001  | <.001  |
| Triglycerides                    | .122   | .034   | <.001  | .074   | <.001  | .008   |
| Glucose                          | .132   | .223   | <.001  | <.001  | <.001  | <.001  |
| Hematocrit                       | .008   | .022   | .007   | .003   | .0299  | .0985  |
| Decrease in total physical activity | .034   | .176   | .008   | .028   | <.001  | <.001  |
| Decrease in handgrip strength    | .098   | .042   | .005   | .006   | .002   | <.001  |
| Decrease in food intake          | .078   | .333   | .002   | <.001  | .035   | .006   |
| Discomfort symptoms              | .212   | <.001  | <.001  | .005   | <.001  | <.001  |
| Decrease in intruder-resident paradigm | .212  | .031   | .185   | .023   | .024   | .008   |
| Decrease in forced swimtest      | .061   | .018   | <.001  | <.001  | .009   | <.001  |

The results represent statistical differences observed between the mean values of the different groups. Body weight loss and gastrocnemius (GSN) weight loss calculations are described in the Methods section. Serum amyloid A (SAA) was expressed in ng/mL, IL-6 was expressed in pg/mL, albumin was expressed in g/dL, Triglycerides were expressed in mg/dL, glucose was expressed in mg/dL. Hematocrit was calculated as percentage of packed cell volume to total blood volume. Total physical activity (TPA) was referenced to the control group (TPA = TPA of TB group at sacrifice/mean TPA of the control group at sacrifice) × 100). Handgrip strength or grip force (GF) was measured in g/g initial body weight and the parameter was referenced to the control group (GF = GF of TB group at sacrifice/mean GF of the control group at sacrifice) × 100). Food intake (TFI) was referenced to the control group (TFI = F of TB group at sacrifice/TFI of the control group at sacrifice) × 100). Discomfort symptoms are described in Table 1. The intruder-resident paradigm and forced swim test were evaluated using a rating scale established to determine the level of interest of the resident-intruder and the time during which the animals were trying to escape from the water, respectively (Table 1).

In summary, the present investigation represents an extension of the cachexia score developed for humans: a quantitative design for classification of the cachectic syndrome in experimental animals. It is interesting to note that, as in humans, four groups of cachectic animals were established, thereby confirming the parameters and measurements involved in this study. The importance of ACASCO becomes clear when considering that determination of the stage of cachexia present in the animals at a particular time point is of the utmost importance to preclinical testing of either drugs or multimodal approaches. The therapy necessary for the treatment of early cachexia is obviously quite different from that of an advanced cachectic syndrome. This fact, within the limitations of extrapolating animal data to the human condition, may be very useful in the near future in the design of different therapeutic approaches for human cancer cachexia.

This classification system also allows monitoring of alterations that occur in the animal. One of the characteristics that makes the system very useful in research is its capacity to evidence the first symptoms and signs of the cachectic syndrome produced by the
This should enable the initiation of anti-cachectic treatment in the early phase of the syndrome, preventing it from reaching an advanced stage. Despite the advantages of the classification system presented here, we are aware of the limitations of our study. These are mainly based on the fact that the scoring scale design is adapted to three established experimental models, and this limits the use of the scale in other experimental models of cachexia; bearing this in mind, further research is needed to validate its applicability.

**FIGURE 1** Conglomerate analysis of the different tumor models. A, Yoshida AH-130 ascites hepatoma model. B, Lewis lung carcinoma model. C, Colon 26 carcinoma.

**FIGURE 2** Correlation of ACASCO with tumor weight. A, Yoshida AH-130 ascites hepatoma model (cells $\times 10^6$). B, Lewis lung carcinoma model (mg). C, Colon 26 carcinoma (mg).
5 | CONCLUSIONS

The results obtained allow us to conclude that the score system will be very useful as an endpoint in pre-clinical studies involving therapeutic strategies for cancer cachexia. The potential benefit of the use of ACASCO relates to establishing the primary endpoint in pre-clinical cancer cachexia drug evaluations.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTION

Each author has participated sufficiently, intellectually or practically, in the work to take public responsibility for the content of the article, including the conception, design, and for data interpretation. All authors have read and approved the final manuscript.

ETHICAL STATEMENT

All animal manipulations were made in accordance with the European Community guidelines for the use of laboratory animals. They were cared for in compliance with the Policy on Humane Care and Use of Laboratory Animals (ILAR 2011) and in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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