Author's response to reviews

Title: Molecular, cellular and physiological characterization of the cancer cachexia-inducing C26 colon carcinoma in mouse

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Author's response to reviews: see over
To the Editor,

Attached please find the revised version of our manuscript entitled: “Molecular, cellular and physiological characterization of the cancer cachexia-inducing C26 colon carcinoma in mouse” (by Paola Aulino, Emanuele Berardi, Veronica Cardillo, Emanuele Rizzuto, Barbara Perniconi, Carla Ramina, Fabrizio Padula, Enrico P. Spugnini, Alfonso Baldi, Fabio Faiola, Sergio Adamo and Dario Coletti), MS: 1918516035306890.

We would like to sincerely thank the Reviewers, who pinpointed specific issues to be addressed to improve the clarity and strength of our manuscript. In general, both Reviewers acknowledged that our paper deals with a matter of unquestionable clinical relevance. However, both Reviewers asked for revisions that required further experimental work. We think that, by following the Reviewers’ indications, we have greatly improved the quality of our manuscript. This led to the addition of novel data, now shown in Table 1 and in the revised forms of Figures 2, 3 and 6.

Below please find the responses to the specific points addressed by the Reviewer (original comments are cited in blue).

Reviewer #1 (Remarks for the Author):

1. It is not clear how much of the data presented in this manuscript are novel (Figures 1, 2, 3 and 6), since they appear to be a new characterization of the well-defined C-26 cancer cachexia model.

As stated by the other Reviewer (see below) “the C-26 is an established model of cancer which has been poorly described from the point of view of cancer-associated cachexia.” We have now better explained this circumstance at the end of the introduction, where we
summarized the content and relevance of our major findings in the context of the background literature. For what concerns the figures mentioned by the Reviewer:

- Fig. 1b and c: the histology of the C26 tumor has been published in 1975 (Corbett et al., Cancer Research 35, 2434-39). Since then the tumor has been variably called carcinoma or adenocarcinoma throughout the literature, without any experimental support. It was essential to show the histology to demonstrate the undifferentiated state of the tumor, which correlates with malignancy and other fundamental features of this cancer.

- Fig. 2a: the kinetics of muscle growth were published in 1990 (Tanaka et al. Cancer Research 50:2290-95). Since we noticed that tumor growth slowly changes with tumor generations (passages in vivo), we found very important to replicate the data twenty years later.

- Fig. 3a, b, c and e: the extent and kinetics of body and organ weight loss in response to tumor load have been reported in a scattered fashion throughout the literature. However, it was important to reproduce these data in order to correlate them with the progression of tumor growth in the same experimental settings. Indeed, this led to a more detailed discussion (solicited by Reviewer #2, see below) of the results.

- Fig. 6a: the upregulation of Atrogin-1 was previously reported.

All other data are original and have never been published to the best of our knowledge. From all the above it is evident that only a minor part of the data shown in our manuscript has been previously reported and that we reproduced such data to compellingly address significant, scientific questions which remained open up to date.

2. Data shown in Figures 4 and 5 are insufficiently described in terms of experimental detail, interpretation, and significance. All aspects should be expanded in order to fully appreciate the contribution of these data to the literature.

The data have been further described in the Results and extensively discussed throughout the Discussion in relation to the literature and to other results of this manuscript.

3. It is not clear how the use of a C-26 tissue transplant provides a more useful model than the well-established C-26 model used by many groups in the field.

We think that the use of a fragment of tissue as opposed to a cell suspension is a major methodological variable concerning this tumor model. From an analysis of the literature,
from our direct experience and from anecdotic reports by colleagues using the C-26 model, we were convinced that using the tissue transplant approach represents a more reliable mean to reproduce results, as highlighted by the low variability shown in the current version of Figure 2a (tumor growth kinetics, mean values ± SEM, the latter included in the revised version). We speculate that, with our approach, all the tumor cells remain in place and are exposed to the same niche, thus leading to a highly reproducible output. Indeed, providing a reference for the variability of tumor growth is a major reason supporting the publication of this kinetics curve, which was originally reported (by us, as well as by Tanaka) without indication of the standard errors.

Minor Essential Revisions:
1. Not all abbreviations are shown.
We spelled out all the acronyms and included them in the Abbreviation list.
2. There are several typos.
We corrected several mistyped words.
3. The literature is superficially described.
The need to keep the length of the manuscript within acceptable standards and the sentence “Please avoid excessive referencing”, in the authors’ instructions, forced us to limit both the number of references and the description of their content. However, we have increased the number of cited papers (additional citations were needed for the wider Discussion of the manuscript in the revised version) trying at the same time to highlight the most relevant aspect of each cited article.
4. Proliferation and apoptotic indices inadequately described.
We improved the description of the proliferation and the apoptotic index in several sections: Materials and Methods, Results, Figure legends.
5. Timing of tissue isolation in some experiments is not discussed.
The text has been fixed accordingly throughout the manuscript.

Reviewer #2 (Remarks for the Author):
[...]
This tumour model has been used for approximately 30 years, although very few papers have considered the consequences of its growth from the point of view of cachexia, so the points
concerning this question are particularly outstanding. [...] major points to be considered concerning the results presented.

The maximum cachectic effect seems to be achieved on day 16 (Figure 3b), which corresponds to the beginning of the exponential growth phase (Figure 2a). This is not an usual finding in other cachexia models, where cachexia progresses with tumour growth. This needs some clarification or comment.

The point raised by the Reviewer is of great importance and it has been extensively discussed in the second paragraph of the Discussion.

It would be useful to express the statistic variability of the data present in Figure 2a.

The authors state that body weight loss is largely explained by muscle wasting, also indicating that numerous skeletal muscles were affected. However they do not shown these important data whereas Figure 3d shows the weights of liver, kidney and heart, which are organs no affected by tumor growth. From the point of view of a characterization of a cachectic model, the data of individual skeletal muscles and also white adipose tissues depots along tumour growth are necessary in order to evaluate the cachexia status of the animals.

We totally agree with the Reviewer. The weights of the muscles analyzed have now been included in Table 1. Pictures demonstrating the wasting effects of tumor load on the subcutaneous fat pads have been included in Figure 3 (now, as panel “d” of this figure). These results have been presented and discussed, with major modification in the Results and Discussion sections.

I can understand the suitability of EDL in such functional studies. However, because different muscles can be differentially affected by cachexia, it could be necessary to extent such studies to other muscles with different fiber composition.

We found the point very interesting. Thus, we extended the functional analysis to the Soleus, a muscle with a similar geometry to the EDL but with different physiological properties. The new data are now included in Figure 6c. As a matter of fact, the Soleus seems to behave
differently from the EDL also in pathological conditions. This, along with the atrophy measured on specific fiber type populations, allowed extensive discussion of the implications. Thus, the Discussion has been significantly remodeled and extended.

For all the above we think we addressed all the criticisms raised by the referee and we hope that our revised manuscript can be found suitable for publication on BMC Cancer.

Kind regards,

Sergio Adamo, M.D.
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