EMBO Molecular Medicine

Microbiota Dysbiosis influences Immune System and Muscle Pathophysiology of Dystrophin Deficient Mice

Andrea Farini, Luana Tripodi, Chiara Villa, Francesco Strati, Amanda Facoetti, Guido Baselli, Jacopo Troisi, Annamaria Landolfi, Caterina Lonati, Davide Molinaro, Michelle Wintzinger, Stefano Gatti, Barbara Cassani, Flavio Caprioli, Federica Facciotti, Mattia Quattrocelli, and Yvan Torrente
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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

14th Jun 2022

Dear Prof. Torrente,

Thank you for the submission of your manuscript to EMBO Molecular Medicine, and please accept my apologies for the delay in getting back to you, which is due to the fact that I had difficulties securing referees with the right expertise, and that one referee needed more time to complete his/her report. We have now heard back from the three referees who evaluated your manuscript.

As you will see from the reports below, they all acknowledge the potential interest of the study, however they also have serious and partially overlapping concerns and agree that the manuscript is too preliminary at this point (inadequate controls, inconsistencies, quantification, statistics, ...).
As addressing all the referees' comments would require a lot of additional time and effort and considering that at EMBO Press we encourage a single round of revisions in a reasonable time frame, I am afraid that we cannot offer to consider the manuscript further.

Given the potential interest of the findings, we would, however, be willing to consider a new manuscript on the same topic if at some time in the near future you obtained data that would considerably strengthen the message of the study and address the referees concerns in full. To be completely clear, however, I would like to stress that if you were to send a new manuscript this would be treated as a new submission rather than a revision and would be reviewed afresh, in particular with respect to the literature and the novelty of your findings at the time of resubmission. If you decide to follow this route, please make sure you nevertheless upload a letter of response to the referees' comments.

I am sorry that I could not bring better news this time and hope that the referee comments are helpful in your continued work in this area.

Yours sincerely,

Lise Roth

Lise Roth, Ph.D
Senior Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

In this work, the authors study the influence of the microbiota on the pathology of Duchenne muscular dystrophy and conclude that 1) dystrophic mice show an age-dependent dysbiosis; 2) diminishing microbiota reduces inflammation and fibrosis while impairing muscle function; and 3) introducing eubiotic miocrobiota to mdx mice reduces inflammation and improves muscle function. In principle, this is study is of special relevance to the neuromuscular field. I commend the authors for the large amount of data generated and the promising results, which have great potential for translation into the clinic. However, I believe that the manuscript has some serious drawbacks that preclude its publication without thorough editing and revision.

1) I found the manuscript very difficult to follow. I strongly suggest the authors to focus their argumentative line cutting back on non-essential data and reinforcing key data.
- Including one or two models with schematics and pathways would help readers follow the study.
- The rational for performing some experiments or analysis is unclear or not strong enough (i.e., lines 220-232, 398-403, 422-425...). Also, there is little clarity in the data explanation (i.e. Fig 4) and interpretation of some results, with some contradictions and confusing statements (i.e., lines 191-217; 385-387;). There are some pieces of data with little relevance; data that is mentioned but not shown in the figures (line 185, line 342), and results that are not even mentioned in the text and should, therefore, be eliminated.
- There are packages of experiments lacking proper controls (i.e., WT data) or in which analyses were performed in a different way, hindering proper comparison and data interpretation (i.e., Fig. EV1, with different proteins analyzed in 3m and 9m mdx mice; Fig.4).
- Figure legends and methods should help further clarify the text, with more detailed information (now lacking in this version).
- In some places, the authors seem to confuse activation/inhibition with upregulation/downregulation (i.e., mechanism of action of ONX-914, line 372, lines 398-400). I could not access or find "supplementary file 3 and 4, and supplementary material, lines 321, 332, 764...)

- It would be helpful to explain the significance of quantifying the different CD+ cell groups.

2) Methods: The methods need to be described in more detail, in order to make them more comprehensive: In particular there is little or no information about tissue processing for each technique, sex of animals used, and efficacy of qPCR primers. Also, GAPDH is not a suitable endogenous for qPCR analysis in mdx mouse tissues (doi: 10.1371/journal.pone.0211384), so a different one or better yet, a normalization factor should be used instead. Furthermore, when using de delta-delta-Ct method in qPCR, similar primer efficiency should be verified using standard curves.

3) Statistical analysis: I find the data visualization and statistical analysis in this paper very problematic. I agree with the authors using dot plots as an optimal way to represent the data, since it is very visual and helpful to better understand the data. However, it is not clear from the analyses performed or the figure legends what does each dot represent. Ideally, each dot should represent a distinct biological sample (i.e. mice), not images, as detailed in Fig. 5E, 5H. However, throughout the manuscript, the dots shown in the graphs hardly match with the number of mice specified in the figures, so the data should be reanalyzed, taking this issue into account. Also, the authors seem to assume that all the data follow a normal distribution, which is not correct. Data should pass a normality test before performing parametric analyses. Alternatively, non-parametric tests may be used. There are experiments that do not seem to have sufficient statistical power (n=2, Fig 6e), so in these cases the sample number should be increased.

4) Figures: The figure legends should clearly state the N and the statistical analysis performed on each experiment. Also, the marks placed on the photos and the abbreviations used.
- Fiber area is not very meaningful, due to high variability in the mdx model. Analyzing the size difference should be more relevant, using N= number of animals for statistical analysis.
- Fiber frequencies (Fig5E, 6B) may be represented as cumulative curves, and averaged within each group. Statistical analysis may be performed this way
- Instead of cells/slice, it should be calculated per area (mm2, Fig4h)

5) Western blot analyses: This is one of the major issues in the manuscipt. The majority of the blots in the manuscript show overexposed bands not suitable for quantification. Some of the blots reveal a high intra group variability that is not observed in the quantification (graphs). The authors mention that 3 experiments were performed independently, but this is not possible when the same samples are used. Please clarify how WBlots analysis was performed and attach the uncropped blots used for quantification.

Minor points:

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The mdx mouse model for Duchenne muscular dystrophy is the standard model used in the DMD research community. While many researchers have begun using mice with a second point mutation, such as the mdx4CV, which helps prevent Dystrophin revertant fibers, the mdx is still a commonly used model for the disease.

Referee #2 (Remarks for Author):

I would like to commend Farini et al. for the effort that clearly went into this manuscript. The work is also timely, as the intersection between gut microbiota and muscle has been of increasing focus in the last few years. Finally, the effect of microbiota, particularly Prevotella, on dysbiosis could be very relevant and interesting if it is also present in DMD patients.

Unfortunately, there are several factors which detract from my enthusiasm for its publication in its current condition. In general, many of the studies are underpowered and many of the changes observed may be attributed to potential litter effects. Further, 3m C57Bl were used as controls for 9m mdx mice without establishing the effects of aging on the microbiota of the C57Bl mice. Finally there are minor revisions needed to word choice and major revisions for references which are incorrectly cited (Sasson for example). Wilcoxon rank sum test was not listed in materials and methods and yet was used in several figures for statistical assessment. It is not clear how this data is being presented with "normalization". Other measurements, such as fibrosis levels, should be conducted using biochemical methods such as hydroxyproline assay in order to eliminate bias or staining effects. It is not always clear which muscles were being evaluated, but in general a 3m mdx mouse will only have significant fibrosis present in the diaphragms relative to C57BL-10 mice. Finally, there are inconsistencies between some of the data and conclusions that

are drawn. For example, Tartaric acid in the 3m old mdx mouse is attributed to Prevotella, and yet is completely absent in 9m mdx mice. Further, there are differences between the reported Cpk levels in 3m mdx mice between figures in this manuscript which is concerning.

The timing of the mouse experiments are unclear in manuscript results as well as the materials and methods. Were 3m/9m old mice ordered from Charles River and then aged 4 weeks for acclimation prior to experiments? Were the FMT experiments literally a 7 day ABX followed by a 5 day experiment.

Overall, the lack of properly powered experiments, large effect size which is rarely observed in mdx mice especially with such short treatment times, and inconsistencies within the manuscript suggest that there is further work to be done before this manuscript is ready for publication in EMBO Molecular Medicine.

Referee #3 (Comments on Novelty/Model System for Author):

The manuscript lack many controls and the n numbers vary between the figures, some n=2 which is unacceptable. While the detailed analysis are relevant and well documented for the animal model used,
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In this manuscript, by Andrea Farini et al, the authors address the possible link between alteration in gut microbiota, immune responses, metabolic changes, and muscle function in an animal model of Duchenne muscular dystrophy (DMD).
The authors report a correlation between the DMD disease features, inflammation, alteration in microbiota and analysis in GF MDX mice or MDX mice treated with antibiotics influences muscle immunity, fibrosis, and atrophy. In addition, the introduction of eubiotic microbiota reduces inflammation and improves muscle pathology and function in the mouse model tested.
Major comments.
The manuscript contains a lot of information that very likely is relevant to the animal model. But the relevance of human DMD is less unclear. Moreover, there are many inconsistencies and a lack of relevant controls reduces the enthusiasm for the manuscript. Specifically
Figure 1. the histopathology is displayed without the control tissue from non-DMD muscle the muscle tissue undergoes extensive changes between 3 to 9 months. This must be displayed. In addition, I don't understand why the n numbers for the different markers measured vary between markers. Generally, one needs an n=4 number to be on the safe side and this is not the case for several of the analysis performed and, in many figures, presented. N=2 is unacceptable
In Figure 2 only the 3 Month analysis is presented. Please include time zero and the nine-month profiles from normal and DMD mice. The same is true for metabolite profiling. Why only show the 3 months data?
Figure 4, the immune profiling, again I'm surprised that no records of neutrophils are documented which is believed to be part of DMD in humans. In addition, how can the number of mice vary between the groups tested? As stated above the n=4 is the minimum and why only report the 3 months and not include the 9 months.
And the additional figures carry the same problem, n number and in some cases 3 months then all of sudden 9 months. The figure has to be presented with all the time points and compared to non DMD control age-matched. Nine-month-old mice undergo considerable changes in many organs including skeletal muscle.
While the authors demonstrate changes in the tetanic force testing, the lack of more, the authors should consider including a Rotarod experiment for example. Again the lack of consistency across the age groups tested is surprising. Often in analysis testing DMD mice, there is an internal control using the diaphragm
which is much less affected at earlier stages. The authors may consider including this as an internal reference (10.3389/fphys.2020.568909, 2020).

Referee #1 (Comments on Novelty/Model System for Author):

In this work, the authors study the influence of the microbiota on the pathology of Duchenne muscular dystrophy and conclude that 1) dystrophic mice show an age-dependent dysbiosis; 2) diminishing microbiota reduces inflammation and fibrosis while impairing muscle function; and 3) introducing eubiotic miocrobiota to mdx mice reduces inflammation and improves muscle function. In principle, this is study is of special relevance to the neuromuscular field. I commend the authors for the large amount of data generated and the promising results, which have great potential for translation into the clinic. However, I believe that the manuscript has some serious drawbacks that preclude its publication without thorough editing and revision.

We thank the Reviewer for the positive evaluation of the manuscript.

1) I found the manuscript very difficult to follow. I strongly suggest the authors to focus their argumentative line cutting back on non-essential data and reinforcing key data.
- Including one or two models with schematics and pathways would help readers follow the study
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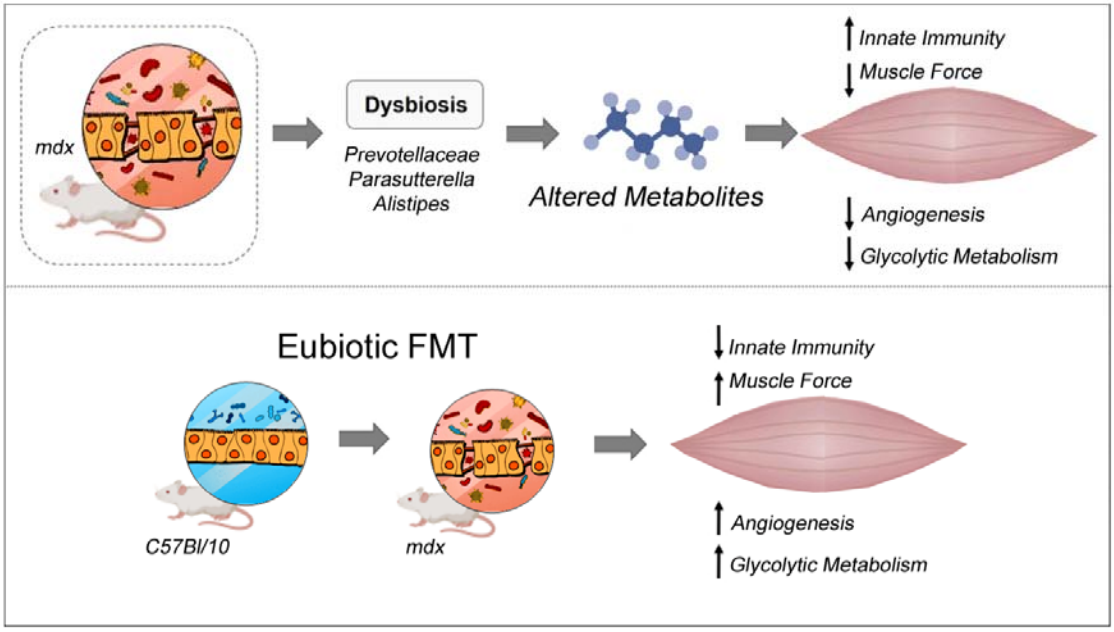
- It would be helpful to explain the significance of quantifying the different CD+ cell groups.

We agree with this referee and really appreciate these comments, which gave us the chance to better support our conclusions. We decided to perform new experiments reinforcing key data with proper comparison and data interpretation on 3m mdx, 3m mdx+ABX and 3m GFmdx and cutting back on non-essential data (i.e. experiments performed with ONX and on 9m mice). We have reworked the text as suggested improving the rational of our analysis. We added the requested corrections in the revised Results, M&M and Legends sections. Moreover, we improved the analysis on eubiotic FMT in 3m mdx providing new data sustaining that restoration of gut microbiota composition improves muscle pathology and function in dystrophic mdx.
A tentative ***Graphical Abstract*** *later be used in case of publication is provided as model for this referee*



Accumulating evidence highlights the importance of the gut-muscle axis. In this study, we demonstrate that the transplantation of gut microbiota from healthy C57Bl/10 mouse to dystrophic mdx mice induces metabolic changes impacting the innate immunity and muscle homeostasis that drive amelioration of muscle pathology and function.

2) Methods: The methods need to be described in more detail, in order to make them more comprehensive: In particular there is little or no information about tissue processing for each technique, sex of animals used, and efficacy of qPCR primers. Also, GAPDH is not a suitable endogenous for qPCR analysis in mdx mouse tissues (doi: 10.1371/journal.pone.0211384), so a different one or better yet, a normalization factor should be used instead. Furthermore, when using de delta-delta-Ct method in qPCR, similar primer efficiency should be verified using standard curves.

In agreement with this referee, Methods have been better described taking into account the EMM length limitations. GAPDH is a widely-used reference gene in the mdx mouse. Thank you for drawing our attention to the work of the Dominic J. Wells lab. Respectfully, the data mentioned in this manuscript are limited by the low number of animals tested (only 3 healthy and 3 dystrophic mdx mice for three distinct time-point: 6, 10 and 24-weeks old). That stated, it is interesting to note that the BestKeeper and Normfinder analysis performed by HildyardI et al, demonstrated similar rank of GAPDH between WT and mdx mice at the 10 weeks old time-point which in mdx correspond to progressing disease with widespread degeneration and compensatory regeneration. We are therefore unable to suggest a better alternative normalization factor. For ddCT in qPCR we used reference genes for which the primers efficiencies were previously validated (between 95.2% and 98.3%). All melt curves shown a single prominent amplicon with melt peaks of between 75 and 85°C. This is now indicated in the Methods section.

3) Statistical analysis: I find the data visualization and statistical analysis in this paper very problematic. I agree with the authors using dot plots as an optimal way to represent the data, since it is very visual and helpful to better understand the data. However, it is not clear from the analyses performed or the figure legends what does each dot represent. Ideally, each dot should represent a distinct biological sample (i.e. mice), not images, as detailed in Fig. 5E, 5H. However, throughout

the manuscript, the dots shown in the graphs hardly match with the number of mice specified in the figures, so the data should be reanalyzed, taking this issue into account.

Data have been reanalyzed including new samples. Overall, each dot of the Figures represented distinct biological sample and n referring to number of animals were indicated in the Legends section. For IF quantification, n = 12 confocal images of TA muscle cross-sections were analyzed from each experimental animal used for each protocol. Two independent experiments were performed for RT-qPCR.

Also, the authors seem to assume that all the data follow a normal distribution, which is not correct. Data should pass a normality test before performing parametric analyses. Alternatively, non-parametric tests may be used.

Hypothesis-testing research evaluates specific hypotheses, using rigorous methods to reduce the risk of bias and a statistical analysis plan that has been defined before the study starts. In contrast, exploratory research, often investigates many questions simultaneously with flexibility to develop or test novel approaches and generate theories and hypotheses that can be formally tested later. Both study types make valuable contributions to scientific progress. In addition, it is difficult to do flexible modelling with non-parametric tests, for example allowing for confounding factors using multiple regression, and parametric tests usually have more statistical power than their non-parametric equivalents to detect significant differences when they truly exist.
In our study, non parametric Wilcoxon rank sum tests were used for microbiota diversity analysis whereas parametric tests were performed for immunity and muscle function as there is a long experience and more than 100 published papers in PubMed on this approach in muscle field. In agreement with this referee, we validated non-parametric analysis performing new analysis of RT-qPCR data with kruskal wallis test (now included in the manuscript).

There are experiments that do not seem to have sufficient statistical power (n=2, Fig 6e), so in these cases the sample number should be increased.

The most challenging parts in establishing and maintaining a Germ Free (GF) mouse model is the handfeeding of suckling mice until weaning and maintaining a GF environment. Repeated assessment of the GF status of the model is essential and improved methods for detecting contamination with increased sensitivity and accuracy were performed by the GF animal facility of University of Lisbon. Long-term maintenance of Germ free mdx animals was influenced by many factors including mortality for intestinal bleeding thus reducing the number of available 9m GFmdx (N=2). Besides, increasing the number of these mice is not easy and would take several months. Thus, we decided to perform new experiments reinforcing key data on 3m groups and cutting back on non-essential data on 9m mice.

4) Figures: The figure legends should clearly state the N and the statistical analysis performed on each experiment. Also, the marks placed on the photos and the abbreviations used.
- Fiber area is not very meaningful, due to high variability in the mdx model. Analyzing the size difference should be more relevant, using N= number of animals for statistical analysis.
- Fiber frequencies (Fig5E, 6B) may be represented as cumulative curves, and averaged within each group. Statistical analysis may be performed this way
- Instead of cells/slice, it should be calculated per area (mm2, Fig4h)

We added the requested experimental details in the figure legends. We paid attention on the image format, by adding scale bar on all the pictures and mentioning the corresponding scales in the figure legends. Relative frequencies analysis is now included in the text and figures section. We

apologize for the mistake made indicating cells/slice instead cells/mm2. This is now corrected in the manuscript.

5) Western blot analyses: This is one of the major issues in the manuscript. The majority of the blots in the manuscript show overexposed bands not suitable for quantification. Some of the blots reveal a high intra group variability that is not observed in the quantification (graphs). The authors mention that 3 experiments were performed independently, but this is not possible when the same samples are used. Please clarify how WBlots analysis was performed and attach the uncropped blots used for quantification.

In order to obtain quantitative data from western blots, a rigorous methodology have been used for all antibodies tested as previously described (10.1007/s12033-013-9672-6). In detail, we calculated the dilution factor of samples that is required for protein loading in the quantitative linear dynamic range for each antibody. To exclude over-exposed blot, the blots were exposed multiple times for increasing periods of time to find the optimal exposure and selected when the signal from specific bands is much stronger than any background noise and a short exposure will pick up only the specific signal. This is recommended by all of the published protocols. Furthermore, we selected the most appropriate normalization method based on reference signals obtained either by housekeeping proteins after immunochemical staining or total protein intensity on blotting membranes after total protein staining. Our WB protocols were standardized considering the sensitivity and specificity of the antibody as referred in literature to assure that the reported fold changes of the target protein are not an artifact of reference signal. We need to account for intra group variation between 9m groups (now excluded also because non-essential data). We now focus our manuscript on groups of 3m mice and WB quantification was performed selecting higher quality images obtained from triple technical replicate for each biological samples. The same variation in data was present between technical replicates. The full-size images for every blot are now provided in raw data file.

Minor points:

- It would be helpful to add all the abbreviations used throughout the manuscript to the table, in alphabetical order. Also, abbreviations should be defined the first time that appears, as well as in the figure legends.

We have updated abbreviations as requested.

- CK results should be further disused, and graph should be reformatted to better show WT and mdx CK values

CK graphs have been reformatted as requested

Referee #2 (Comments on Novelty/Model System for Author):

The mdx mouse model for Duchenne muscular dystrophy is the standard model used in the DMD research community. While many researchers have begun using mice with a second point mutation, such as the mdx4CV, which helps prevent Dystrophin revertant fibers, the mdx is still a commonly used model for the disease.

We are agreed with the reviewer considering the mdx as commonly used DMD model and appropriate for this first description of the role of microbiota on muscular dystrophy.

Referee #2 (Remarks for Author):

I would like to commend Farini et al. for the effort that clearly went into this manuscript. The work is also timely, as the intersection between gut microbiota and muscle has been of increasing focus in the last few years. Finally, the effect of microbiota, particularly Prevotella, on dysbiosis could be very relevant and interesting if it is also present in DMD patients.

We are very grateful for the suggestion of this referee.

Unfortunately, there are several factors which detract from my enthusiasm for its publication in its current condition. In general, many of the studies are underpowered and many of the changes observed may be attributed to potential litter effects. Further, 3m C57Bl were used as controls for 9m mdx mice without establishing the effects of aging on the microbiota of the C57Bl mice.

In agreement with this referee and as previously mentioned in referee #1 responses, we decided to perform new experiments reinforcing key data with proper comparison and data interpretation on 3m mdx, 3m mdx+ABX and 3m GFmdx (including FMT in 3m mdx) and cutting back on non-essential data (i.e. experiments performed with ONX and on 9m mice).

Finally, there are minor revisions needed to word choice and major revisions for references which are incorrectly cited (Sasson for example).

Text and references have been revised as suggested.

Wilcoxon rank sum test was not listed in materials and methods and yet was used in several figures for statistical assessment. It is not clear how this data is being presented with "normalization".

Wilcoxon rank sum tests were used for microbiota diversity analysis and performed on raw results. We specified this statistical test in M&M section.

Other measurements, such as fibrosis levels, should be conducted using biochemical methods such as hydroxyproline assay in order to eliminate bias or staining effects. It is not always clear which muscles were being evaluated, but in general a 3m mdx mouse will only have significant fibrosis present in the diaphragms relative to C57BL-10 mice.

Biochemical methods in general are tissue consuming and not informative on tissue structure. We performed Gomori staining in combination with H&E staining to visualize histological muscle fiber morphology and deposition of collagenous connective tissue in muscle sections. The Gomori Trichrome is a more simplified procedure than other more traditional trichromes. Additionally, this procedure is useful in the assessment of the degree of muscle damage and fibrosis in combination with other immunofluorescence approaches. We agree with the Reviewer that diaphragm is much more fibrotic than TA or other muscles in 3m mdx, even though the primary aim of the paper is to show immune-modulation induced by dystrophic microbiota thus fibrosis represent a post-hoc analysis. We had not performed experiment on diaphragm of animals because it is not clear dynamic and steady states of biochemical pathways involved on inflammation and fibrosis of this type of muscle during disease progression in mdx. Moroever, the low amount of tissue available per animal reduces its application for combinatorial analysis including RNAseq, WB, FACS, and force analysis. However, given the uncertainty on diaphragm characterization we have focus our analysis on the tibialis anterior (TA) muscle as the most used model system in mdx.

Finally, there are inconsistencies between some of the data and conclusions that are drawn. For example, Tartaric acid in the 3m old mdx mouse is attributed to Prevotella, and yet is completely absent in 9m mdx mice.

As previously mentioned, we decided to perform new experiments reinforcing key data with proper comparison and data interpretation on 3m groups only.

Further, there are differences between the reported Cpk levels in 3m mdx mice between figures in this manuscript which is concerning.

The CPK values of 3m mdx are the same between graphs; however, the scale is different thus the values look like diverse. CK graphs have been reformatted to better show CPK values.

The timing of the mouse experiments are unclear in manuscript results as well as the materials and methods. Were 3m/9m old mice ordered from Charles River and then aged 4 weeks for acclimation prior to experiments? Were the FMT experiments literally a 7 day ABX followed by a 5 day experiment.

2m old mice were purchased from Charles River, acclimated for 4 weeks and FMT performed after 7 days of ABX treatment. This is now indicated in the M&M section.

Overall, the lack of properly powered experiments, large effect size which is rarely observed in mdx mice especially with such short treatment times, and inconsistencies within the manuscript suggest that there is further work to be done before this manuscript is ready for publication in EMBO Molecular Medicine.

We carried out new experiments reinforcing key data on 3m groups with proper comparison and data interpretation. Moreover, we added the requested corrections in the revised manuscript.

Referee #3 (Comments on Novelty/Model System for Author):

The manuscript lack many controls and the n numbers vary between the figures, some n=2 which is unacceptable. While the detailed analysis are relevant and well documented for the animal model used, it is not entirely clear if all these analyses presented are relevant for the human form of DMD. One key cell type in human DMD are the neutrophils and they are not recorded in this study

We thank this reviewer for this comment, which gave us the chance to better sustain our conclusions. As such, we have strengthened our paper and included additional data that address the reviewer concerns in the new version.

Referee #3 (Remarks for Author):

In this manuscript, by Andrea Farini et al, the authors address the possible link between alteration in gut microbiota, immune responses, metabolic changes, and muscle function in an animal model of Duchenne muscular dystrophy (DMD).
The authors report a correlation between the DMD disease features, inflammation, alteration in microbiota and analysis in GF MDX mice or MDX mice treated with antibiotics influences muscle immunity, fibrosis, and atrophy. In addition, the introduction of eubiotic microbiota reduces inflammation and improves muscle pathology and function in the mouse model tested.

Major comments.

The manuscript contains a lot of information that very likely is relevant to the animal model. But the relevance of human DMD is less unclear. Moreover, there are many inconsistencies and a lack of relevant controls reduces the enthusiasm for the manuscript.

In agreement with the reviewer's comment, we decided to perform new experiments reinforcing key data with proper comparison and data interpretation on 3m mdx, 3m mdx+ABX and 3m GFmdx (including FMT in 3m mdx) and cutting back on non-essential data (i.e. experiments performed with ONX and on 9m mice).

Specifically
Figure 1. the histopathology is displayed without the control tissue from non-DMD muscle the muscle tissue undergoes extensive changes between 3 to 9 months. This must be displayed. In addition, I don't understand why the n numbers for the different markers measured vary between markers. Generally, one needs an n=4 number to be on the safe side and this is not the case for several of the analysis performed and, in many figures, presented. N=2 is unacceptable

Control tissue is now included in Figure 1. In agreement, we increased the n number of biological samples tested to better sustain our conclusions. Concerning low number (N=2) of Germ Free (GF) mouse model, this was related to the mortality for intestinal bleeding observed during long-term maintenance of Germ free mdx animals. Establishing and maintaining a Germ Free (GF) mouse model is challenging on handfeeding of suckling mice until weaning and maintaining a GF environment. Repeated assessment of the GF status of the model is essential and improved methods for detecting contamination with increased sensitivity and accuracy were performed by the GF animal facility of University of Lisbon. Considering the uncertainty to obtain older GF mdx we decided to exclude the 9m GF mdx analysis and provide more convincing data on 3m groups of animals.

In Figure 2 only the 3 Month analysis is presented. Please include time zero and the nine-month profiles from normal and DMD mice. The same is true for metabolite profiling. Why only show the 3 months data?
Figure 4, the immune profiling, again I'm surprised that no records of neutrophils are documented which is believed to be part of DMD in humans. In addition, how can the number of mice vary between the groups tested? As stated above the n=4 is the minimum and why only report the 3 months and not include the 9 months.
And the additional figures carry the same problem, n number and in some cases 3 months then all of sudden 9 months. The figure has to be presented with all the time points and compared to non DMD control age-matched. Nine-month-old mice undergo considerable changes in many organs including skeletal muscle.

The manuscript data are now referring to 3m mice as reported in the new Figure 2 and Figure 4 that were revised accordingly. As evocated by the reviewer the n number was increased in all groups tested and indicated in the figure legends. Additional raw data file is also included. Concerning the immune profiling, while neutrophils constitute the largest population of all white blood cells in the human circulation (45 - 70%; 1,800 - 6,000/µl), in mice, they are rather sparse (10%; 300 - 500/µl) thus madding difficult to explore these cells in spleen and muscle tissues. To overcome this difficulty, other sources are actually used as bone marrow or the peritoneum following the induction of sterile inflammation but deviate from the aims of our work.

While the authors demonstrate changes in the tetanic force testing, the lack of more, the authors should consider including a Rotarod experiment for example.

We agree with this referee about the advantages in measuring muscle performance using functional tests. Better than Rotarod, which is giving more information on motor coordination, we are familiar with Treadmill exhaustion test. However, to avoid influences of the exercise on gut microbiota we decided to perform tetanic force analysis of TA muscle as the primary muscle function outcome. In fact, treadmill exercise could stimulate microbiome changes thus influencing muscle damage, protein synthesis as well mitochondrial biogenesis.

Again, the lack of consistency across the age groups tested is surprising. Often in analysis testing DMD mice, there is an internal control using the diaphragm which is much less affected at earlier stages. The authors may consider including this as an internal reference (10.3389/fphys.2020.568909, 2020).

We read with interest the two recently published papers cited by the reviewer. We are agreed with the reviewer in the fact that diaphragm twitch kinetics remain virtually unchanged with age in mdx mice. Although we recognize that differences in kinetics should better sustain statistical significance of smaller experimental groups compared to previously published force measurements, the use of diaphragm as internal control was not technically feasible in our animal facility and respectfully not appropriate because it is not clear the dynamic and steady states of biochemical pathways involved on inflammation and fibrosis of this type of muscle during disease progression in mdx. Moreover, the low amount of tissue available per animal reduces its application for combinatorial analysis including RNAseq, WB, FACS, and force analysis. However, given the uncertainty on diaphragm characterization we have focus our analysis on the tibialis anterior (TA) muscle as the most used model system in mdx.

15th Aug 2022

Dear Prof. Torrente,

Thank you for submitting your revised manuscript to EMBO Molecular Medicine.
We have now received the reports from referees #1 and #3 who re-reviewed your manuscript. As you will see, while referee #3 is satisfied with your responses to the initial concerns, referee #1 recognizes the significant work that has been done, but also raises some remaining issues that should be addressed before further consideration of your work.
Therefore, we would like you to address this referee's concerns in a new revised version of your manuscript. Please be aware that this will be the last chance for you to address these concerns.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor.

When submitting your revised manuscript, please carefully review the instructions that follow below. We perform an initial quality control of all revised manuscripts before re-review; failure to include requested items will delay the evaluation of your revision.

We require:

1) A .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) Individual production quality figure files as .eps, .tif, .jpg (one file per figure). For guidance, download the 'Figure Guide PDF' (https://www.embopress.org/page/journal/17574684/authorguide#figureformat).

3) A .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) A complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/17574684/authorguide#submissionofrevisions). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/17574684/authorguide#dataavailability).

In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.

6) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.). Please provide exact p values.

7) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at
.

8) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their

respective legends should be included in the main text after the legends of regular figures.
- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc.
- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.
See detailed instructions here:
.

10) The paper explained: EMBO Molecular Medicine articles are accompanied by a summary of the articles to emphasize the major findings in the paper and their medical implications for the non-specialist reader. Please provide a draft summary of your article highlighting
- the medical issue you are addressing,
- the results obtained and
- their clinical impact.
This may be edited to ensure that readers understand the significance and context of the research. Please refer to any of our published articles for an example.

11) For more information: There is space at the end of each article to list relevant web links for further consultation by our readers. Could you identify some relevant ones and provide such information as well? Some examples are patient associations, relevant databases, OMIM/proteins/genes links, author's websites, etc...

12) Conflict of interest: We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary.

13) Every published paper now includes a 'Synopsis' to further enhance discoverability. Synopses are displayed on the journal webpage and are freely accessible to all readers. They include a short stand first (maximum of 300 characters, including space) as well as 2-5 one-sentences bullet points that summarizes the paper. Please write the bullet points to summarize the key NEW findings. They should be designed to be complementary to the abstract - i.e. not repeat the same text. We encourage inclusion of key acronyms and quantitative information (maximum of 30 words / bullet point). Please use the passive voice. Please attach these in a separate file or send them by email, we will incorporate them accordingly.
Please also suggest a striking image or visual abstract to illustrate your article as a PNG file 550 px wide x 300-600 px high.

14) As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts.
This file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. Let us know whether you agree with the publication of the RPF and as here, if you want to remove or not any figures from it prior to publication.
Please note that the Authors checklist will be published at the end of the RPF.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

I look forward to receiving your revised manuscript.

Yours sincerely,

Lise Roth

Lise Roth, PhD
Senior Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

In this work, the authors study the influence of the microbiota on the pathology of Duchenne muscular dystrophy and conclude that the transplantation of gut microbiota from healthy C57Bl/10 mouse to dystrophic mdx mice induces metabolic changes impacting the innate immunity and muscle homeostasis that drive amelioration of muscle pathology and function. I believe that this is a timely study of high relevance to the neuromuscular field and potential translation into the clinic. However, the technical quality of several experiments needs to be improved.

Referee #1 (Remarks for Author):

I commend the authors for the significant effort that clearly went into revising the manuscript, which has greatly helped clarify the argumentative line by focusing on the key data. Unfortunately, there are still some major concerns that remain to be addressed, which preclude publication in its current form.

1) There are experiments lacking proper controls (WT data). See Fig. 6I, Fig. 7, Fig.8, and EV2. Also, metabolomics analysis in Fig.5 should compare ABX treated mdx with non treated mdx mice.

2) In figure 6, ABX and GFmdx analyses were performed in a different way, hindering proper comparison between ABX treated mdx and GF mdx data.

3) Data interpretation: The authors should make an effort to interpret the results obtained with greater rigor. In particular, different results obtained in ABX and GFmdx models were not mentioned or properly interpret. Also, please revise sentences in line 272 (TNFa is unchanged in mdx vs Ctl, and RelB blots are controversial), lines 301-304 (clarify which groups are being compared), lines 313-316 (needs clarification, and results do not confirm reduced fibrosis in GFmdx); line 329-331 (ACTN3 results must be discussed in greater detail since they seem to contradict previous data regarding increased slow fibers and decreased IIb and IIx area in GFmdx); line 373 (TOMM20 WBlot signals in ABX treated mdx look reduced, while data plotted in the graph is not compliant with images). Also, In EV2 analysis of relevant proteins with altered expression in mdx vs WT should be included, such as PSMB8, PSMB9, PTX3, TLF2, TRAF6...

4) qPCR analyses: I remain unconvinced about the use of GAPDH as the sole endogenous gene in a model where metabolism is clearly affected. Adding one or two additional endogenous genes would increase the reliability of the study. In addition, when using the delta-delta-Ct method, a sample should be used as a reference control. This does not seem to be the case in this study, since values are far from 1. WT samples should be included in these analyses and used as controls.

5) Mass spec imaging: quantification and proper statistical analysis are missing (Fig. 1E)

6) While the authors have greatly improved statistical analysis and data visualization clarity in this version, there are some figures in which dots do not represent different biological samples (mice): Fig. 1C, Fig.7E, F... Please, modify accordingly.

7) Western blot analyses: I remain unconvinced regarding the quality of western blots. To me, signal overexposure is evident in PSMB8, PSMB9, TGFb, COXIV, as well as in b-actin blots, which is most concerning given that b-actin is used as a normalizer in Fig. 1F. An easy way to demonstrate signal linearity is to load double and half the protein content of one sample in each blot. Also, band separation of several proteins is not optimal for quantification of specific bands (TGFb, IGF1, SIRT1, RELB) in Fig1 and EV2. Some blots present too much background for proper quantification (i.e., PPARg, ph-P38, HDAC2...), RelB presents a different pattern in Fig1 (doublet) and EV2 (one band) and there seems to be signal inactivation in COXIV blot(EV2). Most importantly, there are clear inconsistencies in signal quantification of several blots, including PSMB8, PSMB9, and TOMM20. Other minor concerns are lack of molecular weights in the blots. Blot images in PSMB8 (n=9 samples, Fig1) and vinculin in EV2B need to be replaced to better show the specific bands. Same endogenous proteins should be used in Fig.1F and EV2.

8) Please, discuss inconsistencies in CK values in mdx in Fig.7L and Fig.8F (2.000 vs 4.000 U/L). It would help to add CK values of WT mice.

9) Figure legends should be added to supplementary figures EV1-EV5.

Minor points:

- Line 232: tocopherol and tartaric acid data are not shown in this version

- Please clarify the sentence in lines 189-190

- When samples are pooled from different experiments, please state in the figure legend the total n used for statistical analysis

- Line 1256 (Fig.1F): ANOVA is not the proper statistical analysis, because there are only 2 groups.

- Line 1280 (Fig.2A): Shannon index is not included in this version

- Fig 2C: relative abundance in % should reach 100, instead of 1.0. Please, check that Fig.2C and 2F are in the same units.

- Fig2H legend: specify the meaning of ** and ***.

- In Fig. 5E, it seems that data with values equal to 0 have not been included in the analysis. Please, state this in the legend or methods.

- Remove redundant sentence in line 255.

- Graph information is missing in Fig4B and Fig5C (y-axis and meaning of dot size and color)

- GAPDH primer sequences seem to be missing in table 2.

- Please, revise grammar (verb tenses, conjugations, typos...)

Referee #3 (Comments on Novelty/Model System for Author):

Its the model for DMD
not much to say
the medical relevance is high

Referee #3 (Remarks for Author):

I have no further comments

Referee #1 (Comments on Novelty/Model System for Author):

In this work, the authors study the influence of the microbiota on the pathology of Duchenne muscular dystrophy and conclude that the transplantation of gut microbiota from healthy C57Bl/10 mouse to dystrophic mdx mice induces metabolic changes impacting the innate immunity and muscle homeostasis that drive amelioration of muscle pathology and function. I believe that this is a timely study of high relevance to the neuromuscular field and potential translation into the clinic. However, the technical quality of several experiments needs to be improved.

Referee #1 (Remarks for Author):

I commend the authors for the significant effort that clearly went into revising the manuscript, which has greatly helped clarify the argumentative line by focusing on the key data. Unfortunately, there are still some major concerns that remain to be addressed, which preclude publication in its current form.

We would like to thank the referee for carefully reading our manuscript and for giving constructive comments.

1) There are experiments lacking proper controls (WT data). See Fig. 6l, Fig. 7, Fig.8, and EV2. Also, metabolomics analysis in Fig.5 should compare ABX treated mdx with non treated mdx mice.

We have followed this suggestion and more experiments including WT have been performed. We also now compare WT vs mdx vs ABX treated mdx in metabolomics analysis.

2) In figure 6, ABX and GFmdx analyses were performed in a different way, hindering proper comparison between ABX treated mdx and GF mdx data.

Although extensive, our data are limited to GF mdx mouse model. In fact, we face many limitations in the analysis of GF mdx mice including lower availability of animals generated through the "INFRAFRONTIER2020 project and microbiome research", in collaboration with the Gnoto/Axenic Facility of the Instituto Gulbenkian de Ciência (ECGnoto network http://www.ecgnoto.eu), thus reducing the number of samples and experiments. In addition, the number of immune cells in GF mdx tend to be less than WT thus the design of multicolor flow cytometry staining panels is much more difficult than it is for mdx or ABX mdx cell samples. We solved these limitations by adapting FACS analysis to the group of experimental animals: deep analysis increasing the number of parameters analyzed by flow cytometry per cell sample in WT vs mdx vs ABX mdx (i.e. naïve, central memory and effector T cells); essential analysis in WT, mdx, GF mdx (i.e. total CD4, CD8 and Tregs). For these reasons, those different set of experiments are commented separately and without comparison between each other. Unfortunately, we are not in the condition to perform new FACS experiments since GF mdx are no longer available and the generation of others GF mdx animals could require additional months/year.

3) Data interpretation: The authors should make an effort to interpret the results obtained with greater rigor. In particular, different results obtained in ABX and GFmdx models were not mentioned or properly interpret.

Also, please revise sentences in line 272 (TNFa is unchanged in mdx vs Ctl, and RelB blots are controversial)

We performed new western blot analysis including WT animals and revise sentences accordingly to the results indicating that TNFa is unchanged and RelB significantly reduced (new Fig. EV2).

lines 301-304 (clarify which groups are being compared), lines 313-316 (needs clarification, and results do not confirm reduced fibrosis in GFmdx); line 329-331 (ACTN3 results must be discussed in greater detail since they seem to contradict previous data regarding increased slow fibers and decreased IIb and IIx area in GFmdx);

These points are now clarified and commented in the text.

line 373 (TOMM20 WBlot signals in ABX treated mdx look reduced, while data plotted in the graph is not compliant with images).

To better sustain our conclusions we performed new westen blot experiments including characterization of TOMM20 expression (see new Fig EV2).

Also, In EV2 analysis of relevant proteins with altered expression in mdx vs WT should be included, such as PSMB8, PSMB9, PTX3, TLF2, TRAF6.

These proteins were relevant for inflammatory pathways related to dystrophic intestine. However, for muscle characterization, we selected 43 markers among the most described pathways affecting muscle homeostasis. Moreover, we included WT samples in all western blots of Figure EV2.

4) qPCR analyses: I remain unconvinced about the use of GAPDH as the sole endogenous gene in a model where metabolism is clearly affected. Adding one or two additional endogenous genes would increase the reliability of the study. In addition, when using the delta-delta-Ct method, a sample should be used as a reference control. This does not seem to be the case in this study, since values are far from 1. WT samples should be included in these analyses and used as controls.

As suggested, we add one additional endogenous beta-actin gene for qPCR analysis thus increasing reliability of our data (see new Fig 7).

5) Mass spec imaging: quantification and proper statistical analysis are missing (Fig. 1E)

For each lipid, the mean intensity measured at 12 positions (sample area of 100x100 μm^2) throughout the colon images are now shown on the right side of Fig.1E.

6) While the authors have greatly improved statistical analysis and data visualization clarity in this version, there are some figures in which dots do not represent different biological samples (mice): Fig. 1C, Fig.7E, F... Please, modify accordingly.

The number of mice is better specified in raw data file accordingly to the Figure legends.

7) Western blot analyses: I remain unconvinced regarding the quality of western blots. To me, signal overexposure is evident in PSMB8, PSMB9, TGFb, COXIV, as well as in b-actin blots, which is most concerning given that b-actin is used as a normalizer in Fig. 1F. An easy way to demonstrate signal linearity is to load double and half the protein content of one sample in each blot. Also, band separation of several proteins is not optimal for quantification of specific bands (TGFb, IGF1, SIRT1, RELB) in Fig1 and EV2. Some blots present too much background for proper quantification (i.e., PPARg, ph-P38, HDAC2...), RelB presents a different pattern in Fig1 (doublet) and EV2 (one band) and there seems to be signal inactivation in COXIV blot(EV2). Most importantly, there are clear inconsistencies

in signal quantification of several blots, including PSMB8, PSMB9, and TOMM20.
Other minor concerns are lack of molecular weights in the blots. Blot images in PSMB8 (n=9 samples, Fig1) and vinculin in EV2B need to be replaced to better show the specific bands. Same endogenous proteins should be used in Fig.1F and EV2.

All western blot analysis shown in Fig 1 and EV2 have been repeated to ameliorate the quality of the blot images accordingly to the points raised by referee.

8) Please, discuss inconsistencies in CK values in mdx in Fig.7L and Fig.8F (2.000 vs 4.000 U/L). It would help to add CK values of WT mice.

It is well accepted that CK levels are extremely variable between mdx mice of the same age (De Luca A, Am J Pathol. 2005; Grounds MD, FASEB J. 2004; Spurney CF, Muscle Nerve. 2009) thus the range of CK values is more indicative than the absolute CK values (Spurney CF, Muscle Nerve. 2009). Our mdx CK values reported in Fig 7L (mean of CK: 2578.5 U/L) and Fig 8F (mean of CK: 4232.7 U/L) are in range of those reported for 12w mdx by First Workshop Report "A Project to Improve How We Advance Duchenne Muscular Dystrophy Therapies to the Clinic" (headed by Partridge T, Spencer M, Grounds MD, Spurney C, Aartsma-Rus A; 10.3233/JND-180324) and by several other scientific reports (CK ≈ 2600 U/L, 10.1172/JCI7866; CK ≈ 2700 U/L, 10.1186/s13395-017-0135-9; CK ≈ 1900 U/L, 10.1371/journal.pone.0016184; CK ≈ 1700 U/L, 10.3390/antiox10081241; CK ≈ 2300 U/L, 10.1096/fj.12-215723; CK ≈ 4500 U/L, 10.1371/journal.pone.0121556; CK ≈ 2200/4100 U/L 10.1016/j.nmd.2011.10.011; CK ≈ 4200 U/L, 10.3389/fphys.2021.649793). According to the suggestion of the referee we included CK values of age-matched C57Bl mice in the new version of Fig. 7L and 8F (n=8).

9) Figure legends should be added to supplementary figures EV1-EV5.

We add figure legends of EV figures in the text.

Minor points:

- Line 232: tocopherol and tartaric acid data are not shown in this version

Line 232 has been corrected accordingly.

- Please clarify the sentence in lines 189-190

Sentences have been clarified accordingly.

- When samples are pooled from different experiments, please state in the figure legend the total n used for statistical analysis.

The total n used per group is now specified in the figure legends.

- Line 1256 (Fig.1F): ANOVA is not the proper statistical analysis, because there are only 2 groups.

This is now corrected in the text.

- Line 1280 (Fig.2A): Shannon index is not included in this version

This is now corrected in the legend of Fig.2

- Fig 2C: relative abundance in % should reach 100, instead of 1.0. Please, check that Fig.2C and 2F are in the same units.

We confirm that now Fig2C and 2F are the same units.

- Fig2H legend: specify the meaning of ** and *.**

This is now specified in Fig2H legend.

- In Fig. 5E, it seems that data with values equal to 0 have not been included in the analysis. Please, state this in the legend or methods.

Values equal to 0 have been included in the analysis of Fig. 4E

- Remove redundant sentence in line 255.

One sentence was related to splenic cells and the other to muscle cells.

- Graph information is missing in Fig4B and Fig5C (y-axis and meaning of dot size and color)

This is now included in the legend of figure 4D.

- GAPDH primer sequences seem to be missing in table 2.

We performed new RT-PCR analysis with beta-actin as housekeeping and primer sequences are now included in table 2.

- Please, revise grammar (verb tenses, conjugations, typos...).

Typos/language errors have been corrected accordingly.

14th Nov 2022

Dear Prof. Torrente,

Thank you for the submission of your manuscript to EMBO Molecular Medicine. As you will see below, referee #1 is now supportive of publication, and I am therefore pleased to inform you that we will be able to accept your manuscript once the following editorial points will be addressed:

1/ Manuscript text:
- Please address the queries from our data editor in the Data edited manuscript file (figure legends). Please remove the highlighted text and only keep in track changes mode any new modification.
- Please remove the "Teaser" line.
- We can accommodate a maximum of 5 keywords, please adjust accordingly.
- We note that you have a table with the list of abbreviations. Please also define them the first time they appear in the text.
- Materials and Methods: please change the section title: "blurb regarding RNAseq".
- The file "supplementary material" may be added to the main manuscript Materials and Methods, or alternatively in an Appendix file.
- Please place the Data Availability section after the Materials and Methods. Please note that primary datasets such as RNAseq data produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/17574684/authorguide#dataavailability).
In case you have no data that requires deposition in a public database, please state so in this section ("This study includes no data deposited in external repositories."). Note that the Data Availability Section is restricted to new primary data that are part of this study.
- Please merge the funding section with the acknowledgements.
- Author contributions: CRediT has replaced the traditional author contributions section because it offers a systematic machine-readable author contributions format that allows for more effective research assessment. Please remove the Authors Contributions from the manuscript and use the free text boxes beneath each contributing author's name in our system to add specific details on the author's contribution. More information is available in our guide to authors.
- Please rename the "Disclosure statement and competing interests" section. We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary.
- Remove the Table legends and "List of material contained in the Supplementary Material" from the manuscript file.

2/ Figures.
- Please indicate in the figures or in their legends the exact p= values, not a range. Some people found that to keep the figures clear, providing a supplemental table in an Appendix with all exact p-values was preferable. You are welcome to do this if you want to.
- Figure legends: Please update the nomenclature for the EV figures (Figure EV1, etc).
- Figure callouts: References for the EV datasets, for Fig. 2F, 5I, 7D are missing in the manuscript file. Fig. 2G should be called out before Fig.3, and Fig. EV2B should be called out before Fig. EV2B. Fig. 8A is referenced in the text, but there is no such figure.
- There is a file with supplementary Figures. As these are the EV figures, the file can be deleted.
- Please add legends in a separate tab for the 4 EV datasets.

3/ Thank you for providing Source Data. Please upload them as one file per figure.

4/ Checklist:
- Please indicate the corresponding author's name (top left corner)
- Reporting: please indicate in which section of the manuscript is the information available
- Please fill in the part "Plants and microbes" - Microbes

5/ Please provide "The paper explained" section: EMBO Molecular Medicine articles are accompanied by a summary of the articles to emphasize the major findings in the paper and their medical implications for the non-specialist reader. Please provide a draft summary of your article highlighting
- the medical issue you are addressing,
- the results obtained and
- their clinical impact.
This may be edited to ensure that readers understand the significance and context of the research. Please refer to any of our published articles for an example.

6/ Every published paper now includes a 'Synopsis' to further enhance discoverability. Synopses are displayed on the journal webpage and are freely accessible to all readers. They include a short stand first (maximum of 300 characters, including space) as well as 2-5 one-sentences bullet points that summarizes the paper. Please write the bullet points to summarize the key NEW findings. They should be designed to be complementary to the abstract - i.e. not repeat the same text. We encourage inclusion of key acronyms and quantitative information (maximum of 30 words / bullet point). Please use the passive voice. Please attach these in a separate file or send them by email, we will incorporate them accordingly.

Please also suggest a striking image or visual abstract to illustrate your article as a PNG/JPEG/TIFF file 550 px wide x 300-600 px high.

7/ As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts.
This file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. Let us know whether you agree with the publication of the RPF and as here, if you want to remove or not any figures from it prior to publication.
Please note that the Authors checklist will be published at the end of the RPF.

I look forward to receiving your revised manuscript.

Yours sincerely,

Lise Roth

Lise Roth, PhD
Senior Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

I do not agree with the authors' methodology of pooling images from different animals to increase N for statistical analysis. However, this is now clearly stated in the manuscript, and, otherwise, I think that the study is sound and thorough

Referee #1 (Remarks for Author):

I now think that the manuscript is acceptable for publication.

The authors addressed the minor editorial issues.

1st Dec 2022

Dear Prof. Torrente,

Thank you for addressing the last editorial issues. I am pleased to inform you that your manuscript is now accepted for publication and will be sent to our publisher to be included in the next available issue of EMBO Molecular Medicine.

Please read below for additional IMPORTANT information regarding your article, its publication and the production process.

Congratulations on your interesting work!

With kind regards,

Lise Roth

Lise Roth, Ph.D
Senior Editor
EMBO Molecular Medicine

EMBO Press Author Checklist

Corresponding Author Name: Professor Yvan Torrente	
Journal Submitted to: EMBO Molecular Medicine	
Manuscript Number: EMM-2022-16244-V2-Q	

USEFUL LINKS FOR COMPLETING THIS FORM
The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January 2022)
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Reagents and Tools Table (Table 1)

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Materials and Methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Materials and Methods
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Materials and Methods
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods and Figure Legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure Legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure Legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Yes	Materials and Methods
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Materials and Methods
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	