**Point by point response**

Editor comments: written in plain black

Author response: written in plain blue

Quotations of the revised manuscript: Written in *cursive red*

**Reviewer #1 (Comments for the Author):**  
  
In this revised manuscript, the authors have added controls including heart failure individuals without TTNtvs. I think the manuscript is somewhat improved by the inclusion of these controls. My remaining comment is that the non-TTNtv individuals have mutations in genes that regulate TTN including and most importantly RBM20 that is a splicing regulator of TTN. The cohort of 7 contains 4 individuals with RBM20 mutations. Also, MYBPC3 mutations are not well-established causative for DCM. The control cohort is too small to make significant claims.

**Author response:** We appreciate this feedback and the recognition of the improvements we have made to the manuscript. Your concerns about the control cohort, identifying a valid control group and the limitations on cohort size are valid and well-taken. We understand the current debate regarding rare variants in *MYBPC3* and their role in DCM. While some studies have implicated MYBPC3 in the pathogenesis of DCM, others find evidence to be limited. Overall, an excess frequency of 0.6% of (non-truncating) rare variants is observed in DCM vs control cohorts (p=0.128) (1). We have answered the comment regarding *RBM20* in our answer below. Overall, the identification of valid controls was a challenge in this study, and while our current control cohort was not perfect, we were still able to identify differences when compared to the TTNtv group.

**Changes made:** In line with this comment and those from reviewer #2 we have modified the conclusions of the manuscript to fit with the study design and sample size.

The first sentence of the (final) conclusion (page 16, last paragraph) has been modified to read: “We present evidence suggesting involvement of skeletal muscle in persons with TTNtv”.

The conclusion of the abstract (page 3) now reads “Mild skeletal muscle involvement was prevalent in patients with TTNtv. The phenotype was characterized by an increased muscle fat fraction, and excessive accumulation of glycogen, possibly due to reduced autophagic flux. These findings indicate an impact of TTNtv beyond the heart.”. The concluding sentence of the manuscript now reads (page 17) “These insights deepen our understanding of TTNtv's impact beyond the heart and underscore the need for studies of potential clinical impact of extra cardiac work-up of patients with TTNtv.”.

We have also revised the end of the first section of the discussion (page 13, second paragraph) to now read “Collectively, these findings suggest that further investigation of the skeletal muscle phenotype in larger cohorts of individuals with DCM due to TTNtv is needed. Such research will enhance our understanding of the phenotype tied to this relatively prevalent cardiomyopathy genotype.”  
Finally, we have modified the text in the central illustration, removing the sentence suggesting neuromuscular work-up of patients.

Overall, I think it’s prudent to mention in the text that RBM20 may not be the best control for this study due to direct effects on TTN splicing by this protein, and RBM20 is expressed in skeletal muscle of humans (see GTex data). Some text edits are necessary to properly describe these limitations of the control cohort. See <https://gtexportal.org/home/gene/RBM20> for skeletal muscle expression.

**Author response:** Thank you for bringing this to our attention. We had originally chosen to include genes based on their protein expression score from proteinatlas.org, where RBM20 is not considered to be expressed in human skeletal muscle tissue (RBM20: <https://www.proteinatlas.org/ENSG00000203867-RBM20/tissue)>. However, we do acknowledge that RNA-expression of *RBM20* has been observed in skeletal muscle tissue and the (potential) effect of RBM20 in human skeletal muscle tissue is not fully elucidated.

**Changes made:** We have added the following section to the limitations section (page 16, second paragraph) “Selecting the most appropriate control group for cardiomyopathy posed a challenge. Patients with variants in *RBM20*, a regulator of titin splicing, were included. While protein expression of *RBM20* has not been identified in skeletal muscle, RNA expression has been reported and the effect in skeletal muscle isn't fully understood. This inclusion could potentially bias our results towards the null hypothesis”

**Reviewer #2 (Comments for the Author):**  
  
The first sentence of the final conclusion "Persons with TTNtv have involvement of skeletal muscle" is too general and too sweeping for the data presented on this small cohort. Please remove this sentence or modify substantially in light of the comments in the next paragraph, and this first preliminary study of a few patients,

**Author response:** Thank you for this comment. We have modified the conclusion as suggested.

**Changes made:** The first sentence of the (final) conclusion (page 16, last paragraph) has been modified to read: “We present evidence suggesting involvement of skeletal muscle in persons with TTNtv”.  
  
Similarly, any conclusions suggesting, much less recommending, evaluation and work up of patients with muscular complaints who have TTNtvs is premature. This study was not designed to evaluate muscle symptoms objectively. The study would have needed investigators and patients interviewed and tested prior to their genetic diagnoses. The statin literature and muscle complaints has taught us the power of suggestion (<https://pubmed.ncbi.nlm.nih.gov/34531021>/; <https://pubmed.ncbi.nlm.nih.gov/33196154>/).

**Author response:** Thank you for this important comment. We agree with the reviewer that this study does not provide evidence which supports systematic neuromuscular work-up of this specific group of patients with muscular complaints. We have in the revised manuscript tempered our statements in the conclusion section.

**Changes made**: The conclusion of the abstract (page 3) now reads “Mild skeletal muscle involvement was prevalent in patients with TTNtv. The phenotype was characterized by an increased muscle fat fraction, and excessive accumulation of glycogen, possibly due to reduced autophagic flux. These findings indicate an impact of TTNtv beyond the heart.”. The concluding sentence of the manuscript now reads (page 17) “These insights deepen our understanding of TTNtv's impact beyond the heart and underscore the need for studies of potential clinical impact of extra cardiac work-up of patients with TTNtv.”. We have also text on the central illustration, removing the sentence suggesting work-up of patients.

In addition, please see changes highlighted to your question below.

Please modify the text at the end of the first paragraph of the discussion accordingly "Even though TTNtv was associated with mild affection of skeletal muscle, this study shows that personalized diagnostic workup in patients with familial DCM and TTNtv is warranted and that cross-specialty involvement in their care should be considered in patients with muscular symptoms. . . ." In contrast, objective weakness or muscle wasting has always needed a neurological evaluation as has been well established in the heart failure and genetic cardiomyopathy guideline literature -- but that was not investigated here and thus is not relevant in a conclusion.

**Author response:** We appreciate the reviewer’s feedback and have modified as suggested.

**Changes made**: We have deleted the text highlighted by the reviewer and added the following two sentences instead (page 13, second paragraph) “Collectively, these findings suggest that further investigation of the skeletal muscle phenotype in larger cohorts of individuals with DCM due to TTNtv is needed. Such research will enhance our understanding of the phenotype tied to this relatively prevalent cardiomyopathy genotype.”

**Reviewer #3 (Comments for the Author):**  
  
This manuscript compares 25 patients with truncating TTN variants (TTNtv), a leading cause of dilated cardiomyopathy (DCM), with the same number of healthy controls to assess the associations of skeletal muscle phenotypes with TTNtv. The comparison showed substantially and significantly higher fat fractions and paraspinal muscles in the TTNtv group as compared to the control.  
  
1. In the abstract: (1a) TTN should be defined when it appears in the first sentence; (1b) xx {plus minus} xx should be noted as mean {plus minus} SD; (1c) Linear mixed effects (LME) modelling; (1d) "Mild, skeletal muscle involvement is prevalent ..." delete comma?

**Author response:** Thank you for this comment!*1a*: The first mention of TTN refers to the gene (spelled out as *TTN*). We have specified that we refer to the gene in the revised manuscript.

*1b-d*: Thank you for these comments. We have revised as suggested.

**Changes made:** The abstract on page 3 has been revised and now reads.1a: “Geneticvariants in *TTN* are associated”, 1b “(11 women, mean±SD age 51±15 years, left ventricular ejection fraction 45±10%)”, 1c “Linear mixed effects modelling” 1d “Mild skeletal muscle involvement”.   
  
2. The nonparametric Wilcoxon-Mann-Whitney test applies to normal as well as non-normal data. Use it throughout to avoid subjective choice of test.

**Author response:** Thank you for this comment. In the original manuscript we did not choose between the Welch’s T-test and the Wilcoxon test at random but based on data-distribution (as evaluated from QQ-plots) and only one of the tests were applied. However, we agree with you in that the Wilcoxon test retains statistical power even in normally distributed data, that the evaluation of data distribution from QQ-plots is not entirely objective and have in accordance with this now applied the Wilcoxon test throughout as suggested.

**Changes made**: We have changed the following sentence in the statistical analysis section (page 8, second to last paragraph) “Comparisons between groups were performed using Fisher’s exact test or Wilcoxon Mann-Whitney rank-sum, as appropriate”. This has also led to changing some of the p-values reported in the manuscript (all very similar to those previously reported).  
  
3. Some details are needed as to how the healthy controls are matched with the patients. Possibility of residual imbalance/confounding should be discussed.

**Author response:** Thank you for this comment. Our matching process was multi-faceted to ensure the best alignment between our healthy controls and the patient group. Specifically, patients and controls were scanned using the same MRI equipment, the same (research) scanning protocol, and controls were included from the general population in a research setting (no clinical indication for muscle MRI existed). Patients and controls were matched individually based on three factors: age (aiming at age within 2 years of the patient), sex, body-mass index (aiming at a BMI within 2 units of the patient). These three factors were chosen since they have previously been found to correlate with muscle fat fraction. While we tried to implement these stringent matching criteria to minimize confounding, we acknowledge that residual confounders can be a problem in this cohort (e.g., dietary habits, smoking, alcohol, physical activity, and previous unrecorded medical history). We have now expanded the Methods section to provide these details and have added a discussion about potential residual imbalance and confounding in the Limitations section of our paper.

### **Changes made**: We have added the following sentence to the limitations section (page 16 second paragraph) “While controls for MRI scans were matched on factors known to correlate with fat fraction, residual confounding might still have affected our results”. In addition, we have added this section to the supplementary methods “*Matching of controls*

Healthy control subjects were matched individually 1:1 to participants with TTNtv. All participants were scanned using the same magnetic resonance imaging equipment, using the same scanning protocol specified below. Controls were included from the general population in a research setting and did not have a clinical indication for muscle testing. Patients and healthy controls were matched based on three factors: age (aiming at age within 2 years of the patient), sex, body-mass index (aiming at a maximal difference within 2 units). These three factors were chosen since they have previously been found to correlate with muscle fat fraction.”

4. Also, limitation about the cross-sectional nature of the study should be discussed.

**Author response:** Thank you for this comment. We have added information on the limitations of the study design to the revised paper.

**Changes made**: We have added the following sentence to the limitations section (page 16, second paragraph) “Finally, the cross-sectional design of the study introduces a selection and information bias and limits our ability to assess how the phenotype evolves over time.”.

**Production Notes (Comments for the Author):**  
Please add the title and legend for the Central Illustration to the figure caption list on the manuscript file.

**Author response:** We have added the title and legend for the Central Illustration as requested.  
  
Please make your changes on the attached document (formatted for production purposes) and use this file when resubmitting. You will still need to upload your previous figures and any supplemental materials as separate files.

**Author response:** Thank you! We have added our changes to this document as requested.