**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*

**Editorial Board:**  
We are very interested in this manuscript as a research manuscript. However, we would request inclusion of some controls with heart failure. If not, please explain.

**Author response**: We thank the editors for their positive feedback and interest in our study. We have included additional controls in the revised manuscript as requested. To include the most proper control group, we chose to aim at including patients with dilated cardiomyopathy caused by likely pathogenic/pathogenic variants in genes not expressed (based on protein expression score) in skeletal muscle tissue. Included controls were patients with a dilated cardiomyopathy phenotype, likely pathogenic/pathogenic variants in *RBM20*, *DSP*, and *MYBPC3* and heart failure in a stage similar to that observed in the TTNtv cohort (relatively mild with mean LVEF 45% and median NT-proBNP 11 pmol/L).

In short, we found patients with TTNtv, overall, had significantly higher fat fraction than the matched controls with non-TTNtv genetic DCM (+1.5%-points [CI: 0.2 to 2.8]). When looking at each muscle group separately, we found TTNtv patients to have numerically higher fat fractions (both unadjusted and if adjusted for sex, BMI and age) across all muscle groups, although not statistically significant. If comparing healthy controls to patients with non-TTNtv genetic DCM, no significant difference in fat fraction of skeletal muscle was observed.

**Reviewer #1:**  
In this original manuscript by Skriver et al, the authors report a characterization of skeletal muscle structure and function in 25 individuals with TTN truncation variants I-band to M-line that cause dilated cardiomyopathy, but have not been shown to have myopathy previously. The authors have now reported on their cohort of TTNtv individuals with a range of heart failure including some status post transplantation and on a range of heart failure medications. The authors tested their hypothesis that TTNtvs could affect skeletal muscle structure and function given that individuals with muscular dystrophies harbor TTN missense variants mostly localized the m-line structural domain. The authors use MRI to quantify fat content, biopsies to quantify myopathic features including by electron microscopy for a subset of 8 individuals. Finally, muscle strength was assessed by a handheld dynamometer in a make-test. Some muscle groups could not be tested because strength values were above the limits of the assay. The major finding of the study is that TTNtv individuals have increased fat deposition in skeletal muscle, with nominal decrements in muscle strength. The potential for myopathy in TTNtv individuals is of significant clinical interest, but has not been reported previously.

The study suffers from major weaknesses particularly the lack of controls including individuals with heart failure but without TTNtvs, and the lack of normal controls particularly for the muscle test portion of the study. The motor strength assay should not rely on historical controls only. Without each variable being tested in the setting of heart failure with normal TTN, the conclusions of the study may be majorly flawed and essentially unsubstantiated. Many of the reported findings could be secondary to heart failure, or other secondary factors such as medications, but independent of TTN functions. As such, the current version of the manuscript is considered to be of low to marginal value for the field.

**Author response**: We thank the reviewer for taking the time to assess our manuscript. We agree that the design of our study has its limitations, not least due to the invasive nature of the study and the relative rarity of the cohort. However, we believe this study contributes some significant findings from a group of patients who have undergone thorough investigation for muscular symptoms and findings, using a multiparametric approach. Overall, we believe the study will be of interest to the field of inherited cardiomyopathies. We agree with the reviewer and editors that the inclusion of controls (other than healthy matched controls) was warranted. To accommodate this, we have included controls with dilated cardiomyopathy in the revised manuscript. In our estimation, patients with dilated cardiomyopathy caused by genetic variants in genes encoding proteins not expressed in skeletal muscle tissue (and in a similar stage of heart failure) would be the best controls to include. While this made the inclusion of controls complicated and led to a relatively low number of controls (n=7), the findings in this cohort is consistent with that previously reported, with more fat replacement of skeletal muscle observed in patients with TTNtv.  
  
**Changes made**: As suggested by the reviewer, we have added a text-section and additional figures, which details our findings, when comparing TTNtv patients to non-TTNtv DCM patients (attached here below)

“***Fat replacement of muscle versus non-TTNtv genetic DCM***

*In analysis, including patients with DCM of other genetic causes (RBM20, n= 4; MYBPC3, n= 2; DSP, n= 1) and similar LVEF (mean LVEF 45.5% vs 46.4%, p =0.66), mean muscle fat fractions were numerically lower in all muscle groups both in absolute terms (calf Δ-3.2%, p = 0.130; thigh Δ-3.2%, p =0.129; back Δ-5.5%, p=0.61) and when corrected for age, sex and BMI (calf Δ-1.6%, p = 0.198; thigh Δ-1.7%, p =0.103; back Δ-3.9%, p=0.23), although not statistically significant (see* ***Suppl Figure 6****). In linear mixed modelling including results from all investigated muscles, fat fraction was found to be 1.5%-points (CI: 0.2 to 2.8, p =0.025) higher in participants with TTNtv compared to non-TTNtv genetic DCM (****Figure 4****).*”

*Figure 4:*****

*Supplemental Figure 6:*

**Reviewer #2:**

-Please clarify what other variant types were included, such as splice site. Were all pathogenic or likely pathogenic variants in TTN included?

**Author response:** Thank you for this comment. The specific variants for each patient included in the project is listed in Table 1. In summary, we included one patient with a splice site variant, while all others were nonsense or frameshift variants (leading to a premature stop). None of the patients were carriers of multiple pathogenic/likely pathogenic variants in *TTN*, and only patients with likely pathogenic/pathogenic truncating variants were included.

**Changes made**: The following sentence in the results section on page 9, last paragraph, referring to table 1 has been revised for clarity “*For information on clinical characteristics and genotypes of individual participants see* ***Table 1****.*”  
  
-Given that this was a study of skeletal muscle manifestations in DCM, why were patients with myopathy excluded?

**Author response:** We agree with the reviewer that this can seem counterintuitive. However, in this study, we sought to investigate the skeletal muscle phenotype of patients followed at family screening clinics for familial cardiomyopathy. To make us able to include a patient cohort, as similar to that observed in other family screening units as possible, we thought including patients previously identified or suspected to have myopathy could potentially lead to a selection bias, and make our cohort less representative of that met in the clinic.

**Changes made**: None at this point.  
  
-Are CK values available on the cohort? EMG studies?

**Author response**: Thank you for this clarifying question. We did measure CK and myoglobin concentrations in plasma and have provided information on these in the revised manuscript. EMG studies were not performed in patients. CK values were within the normal range in most patients.

**Changes made**: We have specifically stated in the methods section that CK was measured in patients on page 7 “*Cardiac assessments included standard transthoracic echocardiography to assess cardiac function and morphology and analysis of blood for cardiac biomarkers, including N-terminal pro-hormone B-type natriuretic peptide (NT-proBNP) and cardiac tropnonins, and creatine kinase and myoglobin*”. We have also updated the reference to the supplementary table containing that information in the revised results section of the manuscript on page 9 (last section): “*For summary data on clinical characteristics, cardiac medications and muscle biomarkers see* ***Suppl Table 1****.*”   
  
-Would manual muscle testing be more sensitive in identifying small differences in ankle flexion and extension strength?

**Author response:** Manual muscle testing is not likely to identify small differences in muscle strength in persons with (relatively) normal strength. Evaluation of ankle flexion and extension using an isokinetic dynamometer, such as a Biodex system, could potentially identify small differences in strength. However, in patients, with mild (perhaps even subclinical) affection of skeletal muscle, a range of other factors (sex, age, body habitus, coordination/ability to recruit muscle fibers for a maximal effort) are likely to make the identification of these differences unlikely.

**Changes made**: In reference to this point and the point by Reviewer #1, we have added the following sentence to the limitations section on page 16: “*Finally, we did not perform muscle strength assessment of normal controls, which may influence the comparative value and interpretation of these values.*”.  
  
-The data on fat content in muscle are interesting but authors do not speculate on the reason for this. They note similar observations in other muscular dystrophies; is this simply a nonspecific dystrophic process?

**Author response:** Fat replacement of skeletal muscle has been identified to be a prominent feature of many skeletal myopathies, in this setting, indicating a dystrophic process leading to muscle degeneration and lower muscle quality. Fat replacement, is primarily thought to be an indicator of cellular processes with accelerated degeneration and regeneration of muscle fibers, but is likely to be influenced by a range of alternative factors, and is unlikely to be attributed to a single common cellular process.

**Changes made:** We have amended the discussion section to include the following section on page 13 (last section) “*Muscle fat fraction assessed by the Dixon technique is a sensitive tool to detect disease progression in myopathies – with higher values indicating advanced muscle degeneration – and can be identified prior to changes in muscle strength or functional tests*”.  
  
-The authors compare the muscle biopsy findings in this cohort to those of recessive titinopathies, but dominant skeletal muscle titinopathies, such as Udd distal myopathy and HMERF may be better comparators since they share the dominant mechanism and milder skeletal muscle disease.

**Author response**: We thank the reviewer for this comment and agree that comparison to dominant titinopathies is relevant. Also, the reference we have included in the manuscript investigates both dominant and recessive titinopathies.

**Changes made**: We have amended the following sentence (page 14 last section) to include all titinopathies (instead of highlighting the recessive titinopathies). “*In our study, the most common myopathic feature on muscle biopsy was internalized nuclei, as previously observed in titin-related myopathies 35. However, while prior studies have reported central cores to be a prominent pathophysiological feature in titin-related myopathy, this was not the case in our study*”.

-In the discussion, authors note that "In dilated cardiomyopathy, allele dropout and haploinsufficiency caused by TTNtv is an accepted disease mechanism 18, 38, 39, found in 14-20% of DCM patients" but note that recent work by Linke and others suggests a dominant negative mechanism may be (also) at play.

**Author response**: Thank you for bringing this to our attention. We have revised the discussion, to reflect that a dominant negative effect of the truncated protein, has also been implicated as contributing to disease onset/progression. We believe the reference below describes the work you allude to, but please advise if this is not the case.

“Fomin A, Gärtner A, Cyganek L, Tiburcy M, Tuleta I, Wellers L, et al. Truncated titin proteins and titin haploinsufficiency are targets for functional recovery in human cardiomyopathy due to TTN mutations. Sci Transl Med. 2021 Nov 3;13(618):eabd3079.”

**Changes made:** The discussion on page 15 (first section) now reads “*While a dominant negative effect of TTNtv caused by intracellular aggregation of truncated titin has been observed, the underlying mechanism of TTNtv is mostly attributed to allele dropout and haploinsufficiency*”.

-In the introduction it is stated that "the presence and degree of muscle involvement in individuals with familial dilated cardiomyopathy caused by heterozygous TTNtv have not been investigated", but at least one other paper reports on the presence of skeletal muscle features in carriers of TTNtv, specifically Rich et al 2020 (DOI: 10.1002/mgg3.1460) found that 18% of of DCM patients with TTNtv or other pathogenic variants also had skeletal muscle manifestations. Authors should review this publication, which reaches similar conclusions regarding the need for multisystem evaluation of persons with TTNtv and other pathogenic variants.

**Author response**: Thank you for bringing our attention to this paper, which investigates the neuromuscular and cardiac phenotype of probands with TTNtv followed at neuromuscular or cardiology clinics. As mentioned by the reviewer the study observed a cardioskeletal phenotype in 7 of 49 families with 6 of these reported to stem from heterozygous truncating variants in the A-band.

**Changes made**: We have referred to the manuscript mentioned above in the introduction of the revised manuscript, on page4 in the following sentence “*Prior studies in patients with other myopathies have found co-involvement of skeletal and cardiac muscles to be common 12–17 and a previous study investigating the health records of patients with TTNtv found clinical indications of co-involvement of skeletal and heart muscle in a subset of families*”.   
  
-Were the three transplanted patients on corticosteroids during this study (which are known to cause myopathy)? If so the analysis for fat content should be censored for these 3 to ensure that they were not the cause of the positive outcomes.

**Author response**:Thank you for this question. All three transplanted patients received corticosteroids. As suggested by the reviewer, we have performed a sensitivity analysis excluding these patients. We have here attached fat fractions at the 6 scan positions, excluding transplanted patients, which show results consistent with those reported for the entire group.

**Changes made:** We have added the figure attached below to the supplementary material of the manuscript and referenced these findings in the results section on page 10 (end of first section):

“*Since we included patients with cardiac transplants, we performed sensitivity analysis excluding these patients and found results to be consistent with those reported above (****Suppl Figure 5****).*”



In light of use of transplanted patients, please confirm that no other drugs known to cause myopathy were used by any of the patients used in this study.

**Author response**: Thank you for this comment. Supplementary table 1 contains all information on cardiac medications of the participants with TTNtv, included in this project. Aside from the transplanted patients, no participants were on drugs known to cause myopathy.

**Changes made**: None at this point