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T1ρ imaging as a non-invasive assessment of collagen remodeling and organization in human skeletal muscle after ligamentous injury

Brian Noehren, Peter Hardy, Anders Andersen, Camille R Brightwell, Jean Fry, Moriel Vandsburger, Katherine Thompson, and Christopher S Fry
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The referees have opted to remain anonymous.

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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.)

Dear Dr Noehren,

Re: JP-RP-2021-281964 "T1ρ imaging as a non-invasive assessment of collagen remodeling and organization in human skeletal muscle after injury" by Brian Noehren, Peter Hardy, Anders Andersen, Camille R Brightwell, Moriel Vandsburger, Katherine Thompson, and Christopher S Fry

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 expert Referees and I am pleased to tell you that it is considered to be acceptable for publication following satisfactory revision.

Please advise your co-authors of this decision as soon as possible.

The reports are copied at the end of this email. Please address all of the points and incorporate all requested revisions, or explain in your Response to Referees why a change has not been made.

NEW POLICY: In order to improve the transparency of its peer review process The Journal of Physiology publishes online as supporting information the peer review history of all articles accepted for publication. Readers will have access to decision letters, including all Editors' comments and referee reports, for each version of the manuscript and any author responses to peer review comments. Referees can decide whether or not they wish to be named on the peer review history document.

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Yours sincerely,

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EDITOR COMMENTS

Reviewing Editor:

Ethics Concerns:
Authors need to confirm the study conformed to the standards set by the Declaration of Helsinki and also provide the appropriate ethics committee reference number for the study.

Comments to the Author:
This manuscript has been considered by two senior expert reviewers. Both were positive about the work and are of the opinion that the manuscript addresses and interesting and important topic and could make an important contribution to the field. However, both have raised some major concerns about the work and have a common opinion that the some interpretation of the data is not appropriate. Both feel the authors at times over interpret the findings, which is not necessary. The authors should focus on the novelty of the work and keep the message simple.

Senior Editor:

Comments to the Author:
Thank you for submitting your work to the Journal of Physiology. Your work has been reviewed by two referees and a review editor (RE) that are experts in the field; both reviewers and the RE find merit in your study and offer specific suggestions for improvement of your report. We look forward to receiving your revised manuscript.

REFEREE COMMENTS

Referee #1:

The relationship between T1rho and collagen is very important in many areas of application of MRI and so I am genuinely excited by this paper. I congratulate the authors on their rather comprehensive study and I have really enjoyed reading the introduction and discussion which I have found very informative.

I am concerned about some of the interpretation of the data which doesn't seem to be logically backed up by the data. I wonder if I am missing something as I cannot understand why different data was presented for different groups in some sections.

Please can you give further details of the MRI sequence, please can you add a pulse sequence diagram. I assume you used on resonance locking- can you state that in the method?

I think that this title 'T1ρ relaxation time is longer in the ACL-deficient limb along with elevated markers of collagen unfolding and content' should read...

'T1ρ relaxation time, and markers of collagen unfolding and content are elevated in the ACL-deficient limb'

Similarly the title 'Variance in T1ρ relaxation time is differentially explained in healthy and injured skeletal muscle' seems wrong. I think it should read...

'Variance in on-res T1ρ relaxation with collagen content '

I do not understand why Fig 5 only shows ACL def muscles (and fig 6 only shows healthy).. why not show both groups on both of these plots (eg with the healthy samples shown in open circles etc)?

It would have been interesting to have also acquired T2 and T1 data to identify how specific (and sensitive) T1rho is in this context. Please can you add this to the discussion

Have you considered off res spin lock to try to gain more information?

I think some error points are required on the fit to the data at some point. It would be good to see at least an example of the T1rho fit.

MINOR POINTS

Personally I feel the paper is rather long and wordy (particularly at the start) but I am not so familiar with this Journal's style.

On page 16 40.3% should almost certainly be written 40% (do you have an error estimate on that?)

Referee #2:

This study is interesting, relevant and focus on relevant human integrative questions. Having said this, and acknowledging the effort and value of doing such a human study, i think that the authors in order to make it suited for acceptance should revise several points of the manuscript.

Overall i think the paper tried to conclude by far more than is actually addressed in the study design, and i would love to support a version of the manuscript that in a more loyal fashion will appreciate the significant finding,namely that a potential relationship exists between T1p imaging and invasive sample staining of ECM in skeletal muscle.

Don't get me wrong, i really like the approach and will support the manuscript, i just am very sceptical towards your attempt to "push" a conclusion way beyond what the experimental model and the methodology can justify. In its present form its one of these manuscripts where one gets slightly annoyed about the constant "biased" way of writing instead of just stating what you found and discuss this in a more open way.

ORIGINALITY:The authors have contributed to earlier immunohistochemically description of human muscle in healthy and ACL-ruptured legs, and in the present study the new thing is that you couple these findings to a specific way of performing MRI. That's new and that's the important thing!! To add ACL'leg to the healthy one is ok, but you are much less aware of what in fact is happening inside this legs muscle, and its certainly not a muscle injury.

DESIGN OF THE MODEL (MUSCLE INJURY): Its very appreciated that you have an internal control in using a ACL-injured extremity and comparing it to a healthy extremity. The weak point in your approach is that you repeatedly indicate that its a "skeletal muscle after injury " (e.g. the title), and try to claim that pathological changes occur in quadriceps muscle of a ACL-injured leg. No specific proof for this is provided, and to be somewhat critical ("the devils advocate") one could claim that the most likely that happens in an ACL injured leg muscle is simply that muscle atrophy occurs. If this is the case a lot of your "increases" in ECM and collagen semi-quantitative parameters are simply due to a relative increased in ECM/collagen due to a loss of contractile muscle. When we see 10-20% increases in your study we need to know what the concomitant reduction in single fibre CSA is. The model as such is ok for atrophy (as would a one legged immobilization model be), but i think its very "far fetched" that this model is a muscle pathology/injury model.

TITLE AND ABSTRACT: To have the expression "human skeletal muscle after injury" in the title is simply misleading. Its even worse in the abstract where the last sentence mentions "injured muscle" - which has nothing to do with the findings and is an example of the "seductive" writing the authors sometimes use.

INTRODUCTION: The authors use a lot of effort to talk about the organization of ECM in skeletal muscle, but don't use their stainings to study e.g. organization of perimysium/endomysium and do not discuss this further or refer to studies that have tried to look at this. The mentioning of changes in skeletal muscle after ACL injury could be explained by atrophy.

METHODOLOGY: The use of MRI T1p is fine although the explanation of "Low", "Medium" and "High" in fig 1 could be much clearer (and data are provided "randomly" in the figure legend).

The use of immunohistochemistry is acknowledged, but instead of appreciating the limitations of performing which at best is a semi-quantification of stainings, the authors throughout the manuscript are - at least in my view - overly optimistic regarding what can be concluded. A little more "humbleness" would have served the manuscript.

The determination of collagen content or GAG content directly could have been performed on the biopsies.

It cannot be ruled out that the correlations found - significant although quite weak - could have been stronger if more accurate quantification of the ECM/collagen content was obtained.

RESULTS: The text of the results is a mixture of data and discussion, and i think at several points the authors comment upon their findings and try to "lead" the reader. E.g. is the title of fig 5 "Variance in T1p relaxation time is differentially explained in healthy and injured skeletal muscle". First i don't like the "biased" title, secondly to use the word "explained" is not justified" and thirdly, its completely unfair to use the wording "injured skeletal muscle". If you would have liked the paper to deal with "muscle injury" you should have studied that.

STYLE OF WRITING: The authors throughout the manuscript write in a - at least in my view - a very self-confident style, where the often try to force there view through, via biased sentences, biased headings of figures and titles, plus using the obtained data (e.g. immunostainings) that are at best indices or semi-quantitative data as very detailed objective findings.

USE OF REFERENCES: The references are at some points very good and classical references are used and ref to e.g. Lieber et al and their work is useful. It's also understandable that the authors have 20% of all reference to themselves, as they have used the present moden earlier, but i am a little worried about the lack of references that i had expected to see. As the authors are very keen on addressing the problem of "human skeletal muscle after injury" its very strange that although many references are given to atrophy, sarcopenia, mdx etc, only very few references are provided to the study of muscle injury and its recovery after trauma in humans. Several Dutch groups (e.g. Tol) have studied MRI in relation to muscle injury and at least one Danish group (Bayer et al) has studied tissue and MRI in post injury human muscle.

DISCUSSION: The start of the discussion is fine with pointing out the relationship between MRI and direct ECM stainings. So far so good.

But then it starts: "...provide the first direct evidence", and the last sentence in the first paragraph should simply be deleted ("The results of this study..."), you try to conclude that this method (MRI) can be used to follow ECM changes with injury and therapeutic interventions. Be slightly more humble.

In the second paragraph you mention marked ECM changes seen in skeletal muscle in other studies, but you mention Abramowitz 2018 which is on chronic kidney disease and Brashear 2020 which is on mdx mice. Its quite a "stretch" to couple this to the present situation of a completely uninjured human muscle which due to being on a ACL-deficient extremity most likely is atrophied.

Parts of the discussion could be shortened somewhat in that the authors use quite some space in stating that their histology findings fit with what they earlier have found in the same model. If that's the case that part of the study is not so original, its the coupling to MRI that makes the originality in the present manuscript. The last lines of the discussion are very speculative.

END OF COMMENTS

Confidential Review **28-May-2021**

We thank the editor and the reviewers for their thoughtful critique of our submitted manuscript. We have addressed all reviewer and editor comments below with our response appearing in *italics*. Specifically, we note that we have added new experimental data quantifying hydroxyproline abundance of tissue samples, performed additional multivariate analyses to include these new data, and toned down our interpretation and language throughout the manuscript. The results presented in our revised manuscript reflect our new data and additional analyses. We feel the new experimental data, analysis and editing strengthen the manuscript and increase its impact. We hope the reviewers and editors find our revised manuscript suitable for publication.

EDITOR COMMENTS

Reviewing Editor:

Ethics Concerns:
Authors need to confirm the study conformed to the standards set by the Declaration of Helsinki and also provide the appropriate ethics committee reference number for the study.

We have amended the methods section to reflect compliance with the standards set by the Declaration of Helsinki in addition to adding the IRB approval number for this study.

Comments to the Author:
This manuscript has been considered by two senior expert reviewers. Both were positive about the work and are of the opinion that the manuscript addresses and interesting and important topic and could make an important contribution to the field. However, both have raised some major concerns about the work and have a common opinion that the some interpretation of the data is not appropriate. Both feel the authors at times over interpret the findings, which is not necessary. The authors should focus on the novelty of the work and keep the message simple.

We thank the editor for their comments on the contribution of our work. We have edited the language throughout the manuscript, with emphasis on the discussion, to tone down interpretation of the findings we present.

Senior Editor:

Comments to the Author:
Thank you for submitting your work to the Journal of Physiology. Your work has been reviewed by two referees and a review editor (RE) that are experts in the field; both reviewers and the RE find merit in your study and offer specific suggestions for improvement of your report. We look forward to receiving your revised manuscript.

REFEREE COMMENTS

Referee #1:

The relationship between T1rho and collagen is very important in many areas of application of MRI and so I am genuinely excited by this paper. I congratulate the authors on their rather comprehensive study and I have really enjoyed reading the introduction and discussion which I have found very informative.

We thank the reviewer for their kind comments.

I am concerned about some of the interpretation of the data which doesn't seem to be logically backed up by the data. I wonder if I am missing something as I cannot understand why different data was presented for different groups in some sections.

We thank the reviewer for raising this concern. All images were taken within the same set of participants. The data were broken out into their injured limb (labeled ACL deficient) and their non-injured limb (healthy limb). We show that the vastus lateralis T1ρ relaxation time was longer in the in the injured limb. Because the values are significantly different between limbs, we felt it was not appropriate to include both limbs together in the regression analysis. In addition, the healthy limb serves as a genetic control from which we can observe how the relationships between T1ρ relaxation time to markers of collagen content and organization change after ligamentous injury. We have added an additional descriptor that both limbs from the same set of participants were imaged in the methods for magnetic imaging as well as the muscle biopsy section. We have also checked to make sure the same terminology is used throughout the manuscript to describe the two different limbs.

Please can you give further details of the MRI sequence, please can you add a pulse sequence diagram. I assume you used on resonance locking- can you state that in the method?

The reviewer is correct that we only used on resonance locking and have updated the verbiage to reflect this. The MRI sequence we used has already been established with the pulse sequence and block diagram previously published. As we did not develop or create the pulse sequence and the focus of the paper is not on this aspect we felt it was not appropriate to recreate that figure. The full paper on the pulse sequence and block diagram has been published by Signh et al. (which we cite)[1]. We have updated the methods section to state the following "T1ρ images were acquired with a spin lock amplitude of 300 Hz, and ten spin lock hold times of (0/10/20/30/40/50/60/70/80/90ms), using a 4 shot, segmented, gradient echo acquisition (TR of 5.8ms, TE 2.5ms, α=10°, BW=560 Hz/pixel, Nex = 2) and shot TR = 1000ms. The spin lock preparation included a B1 and B0-compensated spin lock preparation pulse followed by a chemical shift selective fat saturation pulse. The sequence employed centric phase encoding to provide exclusive T1rho weighting[1]"

I think that this title 'T1ρ relaxation time is longer in the ACL-deficient limb along with elevated markers of collagen unfolding and content' should read...

'T1ρ relaxation time, and markers of collagen unfolding and content are elevated in the ACL-deficient limb'

We agree with the reviewer and we have edited the subsection title accordingly.

Similarly the title 'Variance in T1ρ relaxation time is differentially explained in healthy and injured skeletal muscle' seems wrong. I think it should read...

'Variance in on-res T1ρ relaxation with collagen content'

We have edited the subsection title to read 'Variance in on-resonance T1ρ relaxation with skeletal muscle collagen content and organization'

I do not understand why Fig 5 only shows ACL def muscles (and fig 6 only shows healthy).. why not show both groups on both of these plots (eg with the healthy samples shown in open circles etc)?

We apologize for the confusion. Given the statistically significant difference in T1ρ relaxation time between healthy and ACL-deficient limbs, we fit separate multiple linear regression models to T1ρ relaxation time for each limb. Since the focus of the paper was not on comparing the explanatory variables across limbs, and because the values were so different between limbs it was felt not to be appropriate to aggregate the data into one model. Rather we fit regression models for each limb so that we could identify different explanatory variables (collagen content and organization) for each limb as each limb was different and thus would have different explanatory variables.

It would have been interesting to have also acquired T2 and T1 data to identify how specific (and sensitive) T1rho is in this context. Please can you add this to the discussion

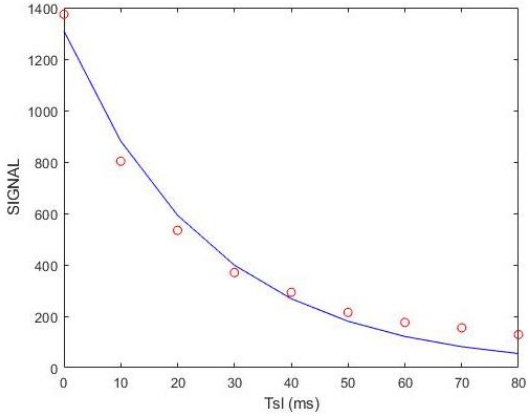
The reviewer brings up a great point and we wish that we had been able to include this additional scan. We have made note of this in the limitations section and as a consideration for future studies.

Have you considered off res spin lock to try to gain more information?

Thank you for this suggestion. This is a great idea however we used did not have the capacity to do it. We hope though that this work stimulates others in the field to consider such experiments.

I think some error points are required on the fit to the data at some point. It would be good to see at least an example of the T1rho fit.

Figure 1 demonstrates the decay of the signal from one pixel in the vastus lateralis in one subject. Before selecting this data, decay curves were calculated from several pixels selected from images acquired on multiple subjects, and the signal decay seen in figure 1 is representative of the general behavior seen in other pixels. The uncertainty in the data points was estimated as the standard deviation of the noise in the background of the raw T1rho images, which varied only slightly from echo-to-echo, and had an amplitude smaller than the size of the symbols. The signal-to-noise ratio of the data was thus quite high. The fit to the data was based on a non-linear, least squares fitting to a model consisting of a mono-exponential decay. A constant term was not included in the model because the SNR was sufficiently high that the non-gaussian distribution of the noise seen at low SNR would not affect the data. The goodness of fit was estimated from the reduced chi squared which averaged about 6 for the signal decay in the pixels reviewed. In the signal decay seen in figure 1, there is, perhaps, evidence of bi- or multi-exponential behavior. There is some interest in multi-exponential fitting to T1rho data in the literature and our histological results, which demonstrate a substantial ECM which would contain water in a very different environment than that within the muscle fiber, might explain the origin of a multi-exponential decay. As we collected only ten echoes because of limited time, we did not believe it was prudent to attempt a fitting of the data to a more complex model. We hope to follow up on this observation by acquiring more data points more closely spaced in time and spanning a greater extent of the T1rho decay curve



in future studies. In addition by establishing the relationship in the current experiment we hope the results propel future research by other groups in the field as well to further tease apart these relationships.

MINOR POINTS

Personally I feel the paper is rather long and wordy (particularly at the start) but I am not so familiar with this Journal's style.

We have edited the length of both the introduction and discussion in an attempt to improve the flow of the manuscript.

On page 16 40.3% should almost certainly be written 40% (do you have an error estimate on that?)

We agree and we have edited the text accordingly.

Referee #2:

This study is interesting, relevant and focus on relevant human integrative questions. Having said this, and acknowledging the effort and value of doing such a human study, i think that the authors in order to make it suited for acceptance should revise several points of the manuscript.

We thank the reviewer for their kind comments, and we have responded to each comment below.

Overall i think the paper tried to conclude by far more than is actually addressed in the study design, and i would love to support a version of the manuscript that in a more loyal fashion will appreciate the significant finding, namely that a potential relationship exists between T1p imaging and invasive sample staining of ECM in skeletal muscle.

We acknowledge the reviewer's comment, and we have toned down our interpretation of the data with less forceful writing throughout the discussion. Editing of verbiage was significant throughout the discussion, and we hope the reviewer can support our newer interpretation of the data, while still underscoring our enthusiasm for this project.

Don't get me wrong, i really like the approach and will support the manuscript, i just am very sceptical towards your attempt to "push" a conclusion way beyond what the experimental model and the methodology can justify. In its present form its one of these manuscripts where one gets slightly annoyed about the constant "biased" way of writing instead of just stating what you found and discuss this in a more open way.

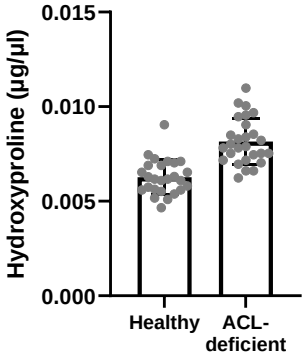
We can appreciate the reviewer's standpoint, and as written above, we have significantly toned down our interpretation throughout the discussion.

ORIGINALITY:The authors have contributed to earlier immunohistochemically description of human muscle in healthy and ACL-ruptured legs, and in the present study the new thing is that you couple these findings to a specific way of performing MRI. That's new and that's the important thing!! To add ACL'leg to the healthy one is ok, but you are much less aware of what in fact is happening inside this legs muscle, and its certainly not a muscle injury.

We agree that the ACL injury does not represent a direct muscle injury, and we have edited our title and other key points throughout the manuscript to make this clearer and reflect the reviewer's comment. Specifically, we have edited the title to reflect the ligamentous nature of the injury, and a similar approach to clarify was taken at several other points in the manuscript. We agree with the reviewer that the novelty of the submitted work is the non-invasive MR imaging within skeletal muscle.

DESIGN OF THE MODEL (MUSCLE INJURY): Its very appreciated that you have an internal control in using a ACL-injured extremity and comparing it to a healthy extremity. The weak point in your approach is that you repeatedly indicate that its a "skeletal muscle after injury " (e.g. the title), and try to claim that pathological changes occur in quadriceps muscle of a ACL-injured leg. No specific proof for this is provided, and to be somewhat critical ("the devils advocate") one could claim that the most likely that happens in an ACL injured leg muscle is simply that muscle atrophy occurs. If this is the case a lot of your "increases" in ECM and collagen semi-quantitative parameters are simply due to a relative increased in ECM/collagen due to a loss of contractile muscle. When we see 10-20% increases in your study we need to know what the concomitant reduction in single fibre CSA is. The model as such is ok for atrophy (as would a one legged immobilization model be), but i think its very "far fetched" that this model is a muscle pathology/injury model.

We agree with the reviewer that the injury is not to the muscle itself, and we have corrected this point throughout the manuscript. To address the comment regarding the semi-quantitative nature of the histology/IHC (which we agree with), we quantitated hydroxyproline content in the same participant biopsies from a small piece of muscle tissue (n=27 [paired limbs]). These new data can be seen below and are added as new Figure 3A. We observed a ~46% elevation in hydroxyproline content in the ACL-deficient limb ($p<0.001$), which we feel adds a degree of confirmation to the differences we observe via histological assessment of collagen in the ACL deficient limb. We acknowledge that histological assessment of ECM/collagen abundance can be influenced by concomitant alterations in muscle fiber size, and we are in agreement for the need of orthogonal approaches to define ECM/collagen limb differences.



Given the hydroxyproline data, we do feel that there is evidence to support the notion of increased ECM content within the muscle of the ACL deficient limb. We fully acknowledge that the degree of ECM expansion is minor-moderate when compared to other more direct muscle injuries or genetic pathologies (dystrophies). That T1ρ relaxation time is sufficiently sensitive to capture these more subtle alterations we feel is a strength of the approach.

TITLE AND ABSTRACT: To have the expression "human skeletal muscle after injury" in the title is simply misleading. Its even worse in the abstract where the last sentence mentions "injured muscle" - which has nothing to do with the findings and is an example of the "seductive" writing the authors sometimes use.

We have edited the title and abstract to remove direct references to 'muscle injury', as well as made these same edits throughout the manuscript to be more clear regarding the ligamentous nature of the injury.

INTRODUCTION: The authors use a lot of effort to talk about the organization of ECM in skeletal muscle, but don't use their stainings to study e.g. organization of perimysium/endomysium and do not discuss this further or refer to studies that have tried to look at this. The mentioning of changes in skeletal muscle after ACL injury could be explained by atrophy.

We acknowledge the excellent point raised by the reviewer – the hierarchical organization of the ECM (endo, peri, epimysium), and how differences at various structural levels could influence relationships with T1ρ times. We agree these measures would add greater depth to our findings, but variation in the biopsies obtained from participants prevented objective analysis of this level of organization across all participants. We have added discussion to this effect in our limitations section. In an effort to validate our histological findings, we performed hydroxyproline assessment as described above, and given the clear elevation in hydroxyproline content between limbs, we feel there is evidence to support our conclusions regarding alterations in the ECM/collagen between limbs.

METHODOLOGY: The use of MRI T1p is fine although the explanation of "Low", "Medium" and "High" in fig 1 could be much clearer (and data are provided "randomly" in the figure legend).

We agree with the reviewer than the language in legend for Figure 1 was unclear and we have edited it for clarity.

The use of immunohistochemistry is acknowledged, but instead of appreciating the limitations of performing which at best is a semi-quantification of stainings, the authors throughout the manuscript are - at least in my view - overly optimistic regarding what can be concluded. A little more "humbleness" would have served the manuscript.

The determination of collagen content or GAG content directly could have been performed on the biopsies.

We agree with the reviewer's assessment regarding immunohistochemistry (semi-quantitative, etc.) – we have toned down our interpretation and added this information to our Limitations paragraph in the Discussion. We have performed analysis of hydroxyproline content within the biopsy samples as an additional measure of collagen content. As we discuss above, the significant ~46% elevation in hydroxyproline content in the ACL-deficient limb supports the histological/IHC results. We have added these data to the manuscript in addition to our multivariate models as an additional explanatory variable. Strikingly, addition of hydroxyproline as an explanatory variable for T1p relaxation time in both the ACL-deficitn and healthy limbs produced a much stronger predictive model. We report these new results in our revised manuscript (new Figures 5B and 6C). We have insufficient remaining tissue for the quantitation of glycosaminoglycan content; we chose to prioritize assessment of hydroxyproline as a measure of collagen abundance given histological differences we observe in collagen-specific staining. Our staining for glycosaminoglycans showed no significant between limb differences.

It cannot be ruled out that the correlations found - significant although quite weak - could have been stronger if more accurate quantification of the ECM/collagen content was obtained.

We acknowledge this point, and we agree with the reviewer. Given unlimited tissue, HPLC/MS/electron microscopy methods could have been employed to fully explore the ECM/collagen. Given limitations with human biopsies, we did not have sufficient tissue for additional assessments. The clinical trial registration of our study states that biopsies are collected for histological/IHC methods only, and we have attempted to minimize the biopsy burden of our participants. We have added discussion to this effect in our limitations section. However, in acknowledgement of the very true points raised by the reviewer, we performed hydroxyproline assessment on subject biopsies. As the reviewer notes, these additional data strengthened the correlations we previously presented in the healthy limb to a sizeable degree (Healthy limb previous $R^2 = 0.26$; revised Healthy limb with hydroxyproline included as an explanatory variable $R^2 = 0.38$).

Additionally, the inclusion of hydroxyproline also strengthened the correlations we previously presented in the ACL-deficient limb to a sizeable degree as well (ACL-deficient limb previous R^2 = 0.40; revised ACL-deficient limb with hydroxyproline included as an explanatory variable R^2 = 0.49).

RESULTS: The text of the results is a mixture of data and discussion, and i think at several points the authors comment upon their findings and try to "lead" the reader. E.g. is the title of fig 5 "Variance in T1p relaxation time is differentially explained in healthy and injured skeletal muscle". First i don't like the "biased" title, secondly to use the word "explained" is not justified" and thirdly, its completely unfair to use the wording "injured skeletal muscle". If you would have liked the paper to deal with "muscle injury" you should have studied that.

We have edited the Results subheadings per the reviewer's comments, as well as removed reference to 'muscle injury'

STYLE OF WRITING: The authors throughout the manuscript write in a - at least in my view - a very self-confident style, where the often try to force there view through, via biased sentences, biased headings of figures and titles, plus using the obtained data (e.g. immunostainings) that are at best indices or semi-quantitative data as very detailed objective findings.

We have toned down our writing style/interpretation throughout the discussion to reflect the suggestions of the reviewer.

USE OF REFERENCES: The references are at some points very good and classical references are used and ref to e.g. Lieber et al and their work is useful. It's also understandable that the authors have 20% of all reference to themselves, as they have used the present moden earlier, but i am a little worried about the lack of references that i had expected to see. As the authors are very keen on addressing the problem of "human skeletal muscle after injury" its very strange that although many references are given to atrophy, sarcopenia, mdx etc, only very few references are provided to the study of muscle injury and its recovery after trauma in humans. Several Dutch groups (e.g. Tol) have studied MRI in relation to muscle injury and at least one Danish group (Bayer et al) has studied tissue and MRI in post injury human muscle.

The focus of the paper was not a direct muscle injury but how an imaging sequence can be used to evaluate muscle with an application given to an indirect injury (ACL tear). No specific area was intentionally left out, but due to brevity and clarity every type of injury and prior work was not covered as much of it was outside the scope of the paper. The work of Bayer et al., highlighted by the reviewer is exceptional and really interesting, however it focused on muscle perfusion whereas the current study focused on ECM/collagen remodeling using a different type of imaging sequence and does not fit within the discussion of fibrosis within muscle[2]. Likewise the work of Tol et al., focused on hamstring injuries and new MRI classification systems which is not directly related to the questions studied in this paper.[3]

DISCUSSION: The start of the discussion is fine with pointing out the relationship between MRI and direct ECM stainings. So far so good.

But then it starts: "...provide the first direct evidence", and the last sentence in the first paragraph should simply be deleted ("The results of this study..."), you try to conclude that this method (MRI) can be used to follow ECM changes with injury and therapeutic interventions. Be slightly more humble.

We have edited the 1st paragraph of the discussion to tone down our interpretation, as well we removed the last sentence.

In the second paragraph you mention marked ECM changes seen in skeletal muscle in other studies, but you mention Abramowitz 2018 which is on chronic kidney disease and Brashear 2020 which is on mdx mice. Its quite a "stretch" to couple this to the present situation of a completely uninjured human muscle which due to being on a ACL-deficient extremity most likely is atrophied.

We agree with the reviewer, and we apologize for our poor choice of wording. We in no way are comparing the morphology we observe following ACL injury to DMD or other models with true fibrotic pathology – the references we cite link changes in the muscle ECM to functional deficits, and our point was to support the concept that alterations in the ECM can have functional ramifications (strength, stiffness), not to compare our results to DMD. We have edited this sentence for clarity.

Parts of the discussion could be shortened somewhat in that the authors use quite some space in stating that their histology findings fit with what they earlier have found in the same model. If that's the case that part of the study is not so original, its the coupling to MRI that makes the originality in the present manuscript. The last lines of the discussion are very speculative.

We acknowledge the reviewer's comment, and we have dramatically shortened/removed the last lines of the discussion. We have reframed parts of the discussion to better highlight the noted novelty of our work – the coupling of MRI with tissue morphology.

1. Singh A, Haris M, Cai K, Kogan F, Hariharan H, Reddy R. High resolution T1ρ mapping of in vivo human knee cartilage at 7T. *PLoS One.* 2014;9(5):e97486.
2. Bayer ML, Hoegberget-Kalisz M, Jensen MH, et al. Role of tissue perfusion, muscle strength recovery, and pain in rehabilitation after acute muscle strain injury: A randomized controlled trial comparing early and delayed rehabilitation. *Scand J Med Sci Sports.* 2018;28(12):2579-2591.
3. Wangensteen A, Guermazi A, Tol JL, et al. New MRI muscle classification systems and associations with return to sport after acute hamstring injuries: a prospective study. *Eur Radiol.* 2018;28(8):3532-3541.

Dear Dr Noehren,

Re: JP-RP-2021-281964R1 "T1ρ imaging as a non-invasive assessment of collagen remodeling and organization in human skeletal muscle after ligamentous injury" by Brian Noehren, Peter Hardy, Anders Andersen, Camille R Brightwell, Jean Fry, Moriel Vandsburger, Katherine Thompson, and Christopher S Fry

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 expert Referees and I am pleased to tell you that it is considered to be acceptable for publication following satisfactory revision.

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I look forward to receiving your revised submission.

If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,

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EDITOR COMMENTS

Reviewing Editor:

Thank you for the revised manuscript and addressing the points raised by the two expert reviewers. Overall, both reviewers feel the paper is interesting and could be important and impactful. However, Reviewer 1 still has some concerns regarding the calibration performed for each limb and would like the authors to be more transparent with these data (please see suggestions from Reviewer 1 to help achieve this). Hopefully the authors see that this will improve the scientific impact of the data and can revise the manuscript accordingly. Reviewer 2 has no further comment.

Senior Editor:

Your revised report has been reviewed by the original reviewers and review editor (RE). Both referees were pleased with the revisions. However, reviewer 1 has (again) raised a question that should be addressed by the authors prior to resubmission. The Journal of Physiology looks forward to receiving your revised manuscript.

REFEREE COMMENTS

Referee #1:

I am still a bit concerned about the fact that different calibrations were done for each limb. I understand the data was very different from each arm but it would at least be good to see the same plots reflected in fig 5 and 6 (maybe with different colours for the two arms on the same plot).. and ideally a single global regression as well as the separate ones.

Also I think a few example fits to the raw data (as shown in response) should be included.. maybe as supplementary materials.

[PEER REVIEW CO-ORDINATOR'S NOTE: the journal doesn't allow supplementary figures or tables. If the authors wish to do as the reviewer suggests, it should form part of the article file, or else comply with journal guidelines]

Referee #2:

The authors have responded adequately to my comments, and I'm fine with the revision.

END OF COMMENTS

1st Confidential Review **23-Jul-2021**

We thank the editor and the reviewers for their thoughtful critique of our submitted manuscript. We have addressed all reviewer and editor comments below with our response appearing in *italics.*

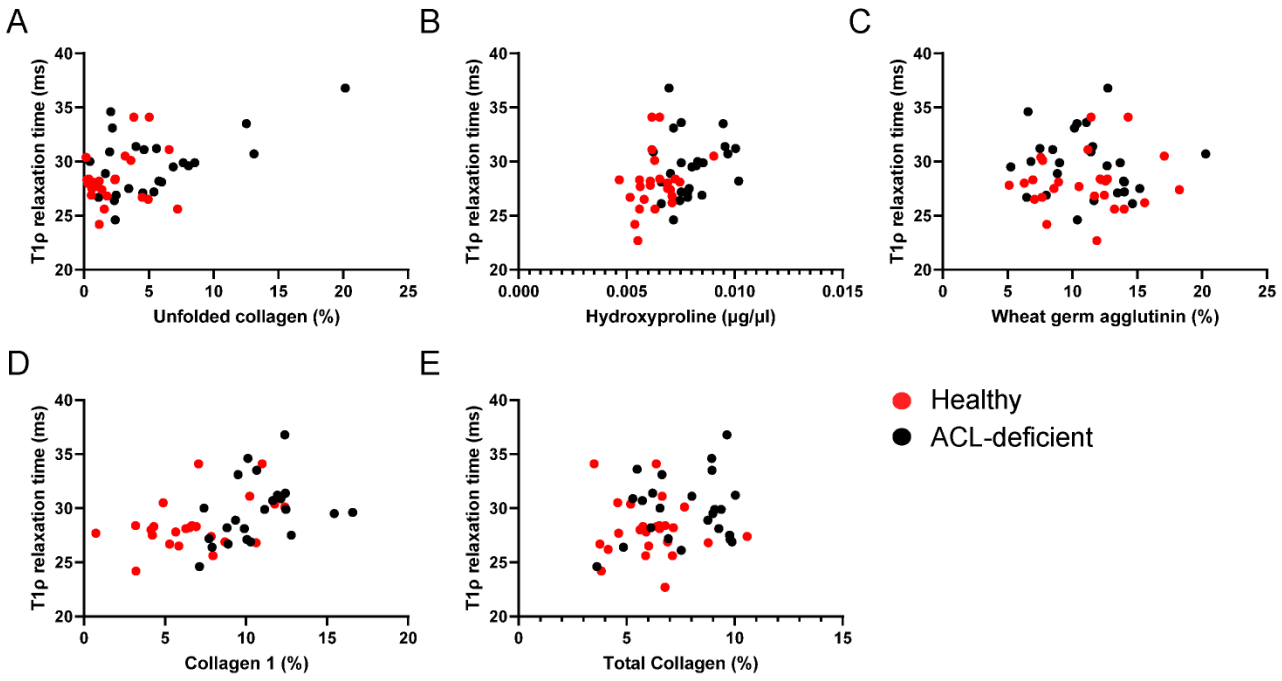
REFEREE COMMENTS

Referee #1:

I am still a bit concerned about the fact that different calibrations were done for each limb. I understand the data was very different from each arm but it would at least be good to see the same plots reflected in fig 5 and 6 (maybe with different colours for the two arms on the same plot).. and ideally a single global regression as well as the separate ones.

We thank the reviewer for their comments. We considered the question of one single model compared to two separate models for each limb, and a single multiple linear regression analysis was not appropriate in this context. Inferences from a multiple linear regression analysis are invalid for this data analysis due to dependence of observations from non-injured and injured limbs within the same subjects. In our case, the two limbs are inherently different and comparing t1rho values across the limbs was not of interest. Thus, injured and non-injured limbs were modeled separately. Statistically, the analogous single model analysis would be a mixed model that allows for correlation within subjects. However, since we found three unique variables for each limb, the mixed model would require over 10 terms to account for the between-limb differences. Thus, the two separate models presented are the appropriate analysis method in this particular case. We've clarified this choice by adding a sentence in the methods to justify the use of two separate models.

We provide below the plots in Figures 5 and 6 (now Figures 6 and 7) with both limbs combined as suggested above.



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[PEER REVIEW CO-ORDINATOR'S NOTE: the journal doesn't allow supplementary figures or tables. If the authors wish to do as the reviewer suggests, it should form part of the article file, or else comply with journal guidelines]

Thank you for this comment, as noted from the peer review co-coordinator the journal does not allow supplementary materials we have included the figure as an example and labeled it as Figure 1.

2nd Revision - Editorial Decision **06-Oct-2021**

Dear Dr Noehren,

Re: JP-RP-2021-281964R2 "T1ρ imaging as a non-invasive assessment of collagen remodeling and organization in human skeletal muscle after ligamentous injury" by Brian Noehren, Peter Hardy, Anders Andersen, Camille R Brightwell, Jean Fry, Moriel Vandsburger, Katherine Thompson, and Christopher S Fry

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 1 expert Referee and I am pleased to tell you that it is almost ready for acceptance. Before formal acceptance, however, you just need to attend to 3 administrative points (see 'Required Items' below).

Please advise your co-authors of this decision as soon as possible.

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I look forward to receiving your revised submission.

If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,

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(2) The Reference List must be in Journal format
Specifically, the references should not be numbered. They should be given in full in the text (e.g. 'Smith et al, 2020) and then listed alphabetically in the reference list.

(3) A Data Availability Statement is required for all papers reporting original data. This must be in the Additional Information section of the manuscript itself. It must have the paragraph heading "Data Availability Statement". All data supporting the results in the paper must be either: in the paper itself; uploaded as Supporting Information for Online Publication; or archived in an appropriate public repository. The statement needs to describe the availability or the absence of shared data. Authors must include in their Statement: a link to the repository they have used, or a statement that it is available as Supporting Information; reference the data in the appropriate sections(s) of their manuscript; and cite the data they have shared in the References section. Whenever possible the scripts and other artefacts used to generate the analyses presented in the paper should also be publicly archived. If sharing data compromises ethical standards or legal requirements then authors are not expected to share it, but must note this in their Statement. For more information, see our Statistics Policy.

EDITOR COMMENTS

Reviewing Editor:

Thank you for making the extra effort in addressing the final reviewer comments and revising the manuscript. It's a very nice paper.

Before final acceptance, please attend to the three administrative points above ('Required Items').

Senior Editor:

Thank for you for submitting your work to the Journal of Physiology and congratulations on the completion of an outstanding study.

REFEREE COMMENTS

Referee #1:

I am now happy with this paper.

_____

END OF COMMENTS

Dear Dr Noehren,

Re: JP-RP-2021-281964R2 "T1ρ imaging as a non-invasive assessment of collagen remodeling and organization in human skeletal muscle after ligamentous injury" by Brian Noehren, Peter Hardy, Anders Andersen, Camille R Brightwell, Jean Fry, Moriel Vandsburger, Katherine Thompson, and Christopher S Fry

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We have edited our paragraph entitled 'Ethical Approval' to be in compliance with these guidelines. Specifically, we have added registration information for our trial.

(2) The Reference List must be in Journal format
Specifically, the references should not be numbered. They should be given in full in the text (e.g. 'Smith et al, 2020) and then listed alphabetically in the reference list.

We have updated the reference style to reflect that of the Journal of Physiology.

(3) A Data Availability Statement is required for all papers reporting original data. This must be in the Additional Information section of the manuscript itself. It must have the paragraph heading "Data Availability Statement". All data supporting the results in the paper must be either: in the paper itself; uploaded as Supporting Information for Online Publication; or archived in an appropriate public repository. The statement needs to describe the availability or the absence of shared data. Authors must include in their Statement: a link to the repository they have used, or a statement that it is available as Supporting Information; reference the data in the appropriate sections(s) of their manuscript; and cite the data they have shared in the References section. Whenever possible the scripts and other artefacts used to generate the analyses presented in the paper should also be publicly archived. If sharing data compromises ethical standards or legal requirements then authors are not expected to share it, but must note this in their Statement. For more information, see our Statistics Policy.

We have added a Data Availability Statement in the Additional Information section of the manuscript. We specifically state: "The data that support the findings of this study are available within the paper and the Supplementary Statistical Summary Document. Additional data are available from the corresponding author upon reasonable request."

EDITOR COMMENTS

Reviewing Editor:

Thank you for making the extra effort in addressing the final reviewer comments and revising the manuscript. It's a very nice paper.

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I am now happy with this paper.

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END OF COMMENTS

Dear Dr Noehren,

Re: JP-RP-2021-281964R3 "T1ρ imaging as a non-invasive assessment of collagen remodeling and organization in human skeletal muscle after ligamentous injury" by Brian Noehren, Peter Hardy, Anders Andersen, Camille R Brightwell, Jean Fry, Moriel Vandsburger, Katherine Thompson, and Christopher S Fry

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Thank you for submitting this outstanding study to the Journal of Physiology.

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