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Multimodal imaging shows fibrosis architecture and action potential dispersion are predictors of arrhythmic risk in spontaneous hypertensive rats

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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 Trew,

Re: JP-RP-2021-281633 "Arrhythmic risk in animal model of hypertensive heart disease predicted by distribution of patchy fibrosis" by Prashanna Khwaounjoo, Gregory Sands, Ian LeGrice, Girish Singh Ramulgun, Anne M Gillis, Bruce Smaill, and Mark Trew

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by two Referees and the reports are copied below.

Please let your co-authors know of the following editorial decision as quickly as possible.

As you will see, in its current form, the manuscript is not acceptable for publication in The Journal of Physiology. In comments to me, the Reviewing Editor expressed interest in the potential of this study, but much work still needs to be done (and this may include new experiments) in order to satisfactorily address the concerns raised in the reports.

In view of this interest, I would like to offer you the opportunity to carry out all of the changes requested in full, and to resubmit a new manuscript using the "Submit Special Case Resubmission for JP-RP-2021-281633..." on your homepage.

We cannot, of course, guarantee ultimate acceptance at this stage as the revisions required are substantial. However, we encourage you to consider the requested changes and resubmit your work to us if you are able to complete or address all changes.

A new manuscript would be renumbered and redated, but the original referees would be consulted wherever possible. An additional referee's opinion could be sought, if the Reviewing Editor felt it necessary. A full response to each of the reports should be uploaded with a new version.

I hope that the points raised in the reports will be helpful to you.

Yours sincerely,

Harold D Schultz
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EDITOR COMMENTS

Reviewing Editor:

The work uses a novel approach to quantify fibrosis and associated arrhythmia risk, raising interesting new hypothesis on how cell connectiveness drives arrhythmia risk. Albeit mostly descriptive, the experimental work is of high quality and interesting to the field. However, both reviewers point out the lack of a non-SHR control group, and i concur. Historical controls are not acceptable for an experimental study as reported here. One age group is likely sufficient, ideally 18 month old normal rats. In addition, the authors need to acknowledge and discuss the limitations and concerns raised by the reviewers, ideally with some new data.

Senior Editor:

Comments for Authors to ensure the paper complies with the Statistics Policy :

show data points in bar graphs

REFEREE COMMENTS

Referee #1:

In this study, Khwaounjoo et al. characterize electrical remodeling and arrhythmia risk in the setting of fibrosis in a rat model of hypertensive heart disease. The investigators optically map Langendorff-perfused spontaneously hypertensive rat hearts at 6, 12 and 19 months and performed microscopic structural analysis. They demonstrate that increased fibrosis was related to decreased conduction velocity and increased anisotropic conduction, action potential duration (APD) and APD dispersion and VT/VF risk. The authors conclude that measures of extent and regional complexity of patchy fibrosis rather than fibrosis density predict arrhythmic risk.

Overall, this is a very interesting study that provide a link between structure and function in the arrhythomogenesis in hypertensive heart disease. My comments are as follows:

1. While this study may be the first to evaluate the relation of fibrosis to electrical remodeling in this animal model of hypertensive heart disease, fibrosis and arrhythmia risk has been evaluated in Langendorff-perfused and in vivo human hearts in myocardial infarction and nonischemic cardiomyopathy and some examples should be cited:

https://pubmed.ncbi.nlm.nih.gov/2345240/

https://pubmed.ncbi.nlm.nih.gov/9669269/

https://pubmed.ncbi.nlm.nih.gov/8325977/

As discussed in de Bakker et al. 1990, the concept of regional complexity of fibrosis rather than estimates of fibrosis density as better predicting VT/VF risk has been raised in myocardial infarction (i.e. surviving myocytes near the infarcted region and "zigzag course of propagation') and should also be discussed in relation to the finding regarding the tortuous fibrosis described in the current study.

2. While connected myocytes was assessed based on proximity, the reason why Cx43 expression was not more extensively assessed should be provided. While Figure 4C details the comparison of connected adjacent cells to fibrosis density, the representative images do not clear provide examples of which adjacent myocytes would be considered connected in the images in Figure 4

3. Could you provide further clarification on how fibrosis was identified on immunohistochemistry. Why was WGA used as cell membranes are also identified?

4. As VT/VF was inducible, were any reentrant circuits of VT able to be mapped to structure?

5. Would refrain from using the phrase "trend was marginally non-significant" and simply that it was non-significant.

6. For the values in Table 1, can more details be provided about how the tissues were analyzed?

7. For Figure 3A, could you clarify why data were binned from 15-165 degrees rather than showing the full 360-degree circumference?

8. Figure 5: were similarly sized areas and locations assessed in the optical maps for each heart? The images in Figure 5A look somewhat disparate with only a scale bar for time. A scale bar for distance should also be provided.

9. While non-hypertensive controls have been previously described as noted in the limitation, a discussion should be provided of how the current study compares to those historical controls. In particular, can insights be drawn on effects of age vs hypertension on the electrical remodeling described?

Referee #2:

Khwaounjoo et al investigate the arrhythmia risk in SHR hearts and relate it to patchy fibrosis with greater resolution and

quantification than previously done. They measure arrhythmia risk by optical mapping of APs in Langendorff perfused hearts, measure longitudinal, transverse and the eccentricity of conduction velocity, VT/VF occurrence and relate these parameters to a cross-sectional analysis of patchy fibrosis on the LV as both increase in severity as a function of age (6, 12 and 18 months old) SHR rats. As previously shown, they confirm that CV decreases in 12 and 18 months old male SHR hearts but include more detailed measurements of longitudinal and transverse CV. VT/VF risk is related to frequency of stimulation and magnitude of voltages applied and measure changes on APD. Patchy fibrosis is measured in clarified tissues using WGA as a marker and point out that the patchiness of fibrosis in the mid-wall of the left myocardium increases with age. These experiments are not innovative and have been routinely done by others but are now done with a focus on SHR hearts. There are, however, major conceptual concerns.

1) The notion that fibrosis, be it on the surface, interstitial or patchy, can account for VT/VF risk has yet to be proven and the present data proposes a guilt by association. This is on on-going controversy and what is needed is data that relates the 'local' fibrosis to local electrical abnormalities.

2) The study ignores a great deal of data that relate electrical dysfunction in SHR rats to changes in ion channel expression, including Na+ channels, L-type Ca2+, lateralization of gap-junctions etc.

3) There are no controls with aged matched non-SHR rats which may also develop patchy fibrosis as in the aged population.

4) There is no data on the nature and structure of the arrhythmias to support the notion that the changes in CV are localized to regions of high-patchiness and to sites where VF is initiated.

5) The authors make several claims about the significance of their study: a) There is increased arrhythmic risk with progression of hypertensive heart disease; this is not new. b) Computational studies attempted to quantitatively relate arrhythmia risk to fibrosis, but thus far failed strictly by considering morphological changes of the tissue. This implies the need to include other changes that occur in HHD to explain their greater propensity to VT/VF.

6) Despite their claims, the authors do not demonstrate that arrhythmia risk is more reliably predicted by measures reflecting the extent of regional patchiness because they do not show controls where non-patchy fibrosis exhibits diminished or equal VT/VF risk.

7) Late gadolinium-enhanced cardiac MRI might not have the spatial resolution to detect complex regional patchy fibrosis to diagnose/predict HHD arrhythmias.

8) Although not essential, it would have been interesting to verify for sex-differences in VT/VF risk and/or patchy fibrosis.

ADDITIONAL FORMATTING REQUIREMENTS:

-Author photo and profile. First (or joint first) authors are asked to provide a short biography (no more than 100 words for one author or 150 words in total for joint first authors) and a portrait photograph. These should be uploaded and clearly labelled with the revised version of the manuscript. See Information for Authors for further details.

-You must start the Methods section with a paragraph headed Ethical Approval. A detailed explanation of journal policy and regulations on animal experimentation is given in Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology by David Grundy J Physiol, 593: 2547-2549. doi:10.1113/JP270818.). A checklist outlining these requirements and detailing the information that must be provided in the paper can be found at: https://physoc.onlinelibrary.wiley.com/hub/animal-experiments. Authors should confirm in their Methods section that their experiments were carried out according to the guidelines laid down by their institution's animal welfare committee, and conform to the principles and regulations as described in the Editorial by Grundy (2015). The Methods section must contain details of the anaesthetic regime: anaesthetic used, dose and route of administration and method of killing the experimental animals.

-Your manuscript must include a complete Additional Information section

â€"Please upload separate high-quality figure files via the submission form.

-You must upload original, uncropped western blot/gel images (including controls) if they are not included in the manuscript. This is to confirm that no inappropriate, unethical or misleading image manipulation has occurred https://physoc.onlinelibrary.wiley.com/hub/journal-policies#imagmanip These should be uploaded as 'Supporting information for review process only'. Please label/highlight the original gels so that we can clearly see which sections/lanes have been used in the manuscript figures.

â€"Your paper contains Supporting Information of a type that we no longer publish. Any information essential to an understanding of the paper must be included as part of the main manuscript and figures. The only Supporting Information that we publish are video and audio, 3D structures, program codes and large data files. Your revised paper will be returned to you if it does not adhere to our Supporting Information Guidelines

-A Statistical Summary Document, summarising the statistics presented in the manuscript, is required upon revision. It must be on the Journal's template, which can be downloaded from the link in the Statistical Summary Document section here: https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics

-Papers must comply with the Statistics Policy https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics

In summary:

-If n â‰¤ 30, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (preferably with data points included) are acceptable formats.

-If n > 30, then the entire raw dataset must be made available either as supporting information, or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

-'n' clearly defined (e.g. x cells from y slices in z animals) in the Methods. Authors should be mindful of pseudoreplication.

-All relevant 'n' values must be clearly stated in the main text, figures and tables, and the Statistical Summary Document (required upon revision)

-The most appropriate summary statistic (e.g. mean or median and standard deviation) must be used. Standard Error of the Mean (SEM) alone is not permitted.

-Exact p values must be stated. Authors must not use 'greater than' or 'less than'. Exact p values must be stated to three significant figures even when 'no statistical significance' is claimed.

-Statistics Summary Document completed appropriately upon revision

-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.

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Confidential Review **11-Mar-2021**

Thank you to the reviewers and the Reviewing Editor for their insightful and considered responses. We address the issues raised in sequence.

Reviewing Editor:

The work uses a novel approach to quantify fibrosis and associated arrhythmia risk, raising interesting new hypothesis on how cell connectiveness drives arrhythmia risk. Albeit mostly descriptive, the experimental work is of high quality and interesting to the field. However, both reviewers point out the lack of a non-SHR control group, and I concur. Historical controls are not acceptable for an experimental study as reported here. One age group is likely sufficient, ideally 18 month old normal rats. In addition, the authors need to acknowledge and discuss the limitations and concerns raised by the reviewers, ideally with some new data.

Thank you for supportive comments and an opportunity to respond to questions and issues raised by you and the reviewers. We revised our manuscript to include new data from 18 month old normotensive rats and additional quantitative data analysis of 10 variables collected across SHR animals. We believe both these additions have strengthened our arguments.

We understand concerns over no age-matched non-hypertensive control data, and we realise this should have been discussed more extensively. The literature shows comprehensively that LV structure, electrical properties and VT/VF risk alter with age in both SHR and normotensive Wistar-Kyoto (WKY) rats. For example:

- By 6 months SHRs are already more susceptible to increased VT risk compared to controls (Tribulova et al, *Gen Physiol Biophys*, 22, 369, 2003; Nguyen et al, *J Physiol*, 594(6), 1689, 2016; Sung et al, *ESC Heart Failure*, ehf2.13013, 2020).
- There are considerable data showing increasing fibrosis with age in SHR vs WKY rats (LeGrice et al, *AJP Heart Circ*, 303, 1353, 2012 - ~ 3, 12, 18 and 24 months; Chan et al, *J Cardiovasc Pharmacol*, 57, 469, 2011 - 3, 6, 9, 12, 15, 18, 21, 24 months).
- There are data confirming increased focal lesions or replacement scar in SHR vs normotensive rats (in addition to the interstitial fibrosis consistent with aging) (Herrmann et al, *Euro Heart J*, 16, 243, 1995 - ~6, 12 and 18 months).
- APD durations in SHR vs normotensive rats separate at around 6 months and are significantly different from that time point on (Chan et al, *J Cardiovasc Pharmacol*, 57, 469, 2011 - 3, 6, 9, 12, 15, 18, 21, 24 months).

These studies are referenced and discussed in the modified manuscript (lines 85-88 Introduction, lines 361-363, 366-373, 389-393 Discussion section 4.1, lines 446-448, 452-455 Discussion section 4.3 and lines 501-509, Discussion section 4.5).

Our primary objective was to determine the extent to which structural and electrical remodelling interact with increasing arrhythmic risk and progression of HHD. We viewed this as a cross-sectional study of the evolution of hypertensive heart disease (HHD) that was best controlled against the youngest cohort. As noted above, divergence of SHRs from normotensive controls is reported at ~6 months and this was selected as the youngest age group. SHRs progress to end-stage heart failure from ~24 months (LeGrice et al, *AJP Heart Circ*, 2012;303:1353) and the oldest cohort was set to 18 months to limit confounding influences introduced by this.

Reviewer 2 confirmed that our optical mapping techniques and electrical recording methods are well established ("...routinely done…"), and we have previously reported on these (Khwaounjoo et al, *Ann Biomed Eng*, 43(5), 1235, 2015). However, a new methodology in our study was the tissue preparation and imaging for structural quantification. From those data we identified regions of aggregated cardiomyopathy throughout the ventricles and obtained animal-specific indices relating

to progression of HHD. We acknowledge that our imaging protocols in optically cleared tissue are novel and that additional imaging data from normotensive animals (WKY) would be an important confirmation of our imaging findings. The literature indicates that normotensive animals will not develop patchy replacement fibrosis as they age (Herrmann et al, *Euro Heart J*, 16, 243, 1995).

We are pleased to have sourced four intact ventricles from 18 month WKY rat hearts immediately after a separate study conducted by colleagues on the atria, also using a Langendorff apparatus. Serial short-axis ventricular slices were cut and then processed, optically-cleared and imaged in an identical manner to our study of SHRs. This arrangement enabled us to complete control studies without sacrificing additional animals and therefore bypass delays associated with the supply of aged WKY rats and in obtaining approval from the University Animal Ethical Committee to extend this study. We have added lines 175-180 in Methods section 2.4. The image data from the 18 month WKY rats show no evidence of patchy replacement fibrosis, in contrast to our findings for 12 and 18 month old SHRs (lines 244-246 Results section 3.1, lines 370-373 Discussion section 4.1 and supporting Image Data: https://doi.org/10.17608/k6.auckland.16829017). We have expanded the author list to recognise the input of our colleagues in the new experiments and the additional imaging and analysis conducted by them.

Prompted by you and the reviewers we have included an additional in-depth multivariable analysis that confirms links between VT/VF risk, structure and APD dispersion (lines 215-228 Methods section 2.6, lines 322-344 Results section 3.4, lines 390-396 Discussion section 4.1, lines 400-402, 417-418, 421-427, 432-436 Discussion section 4.2, and lines 455-461, 469-470 Discussion section 4.3).

We believe these revisions and additions improve the manuscript and strengthen our arguments. We hope that these changes are sufficient to meet the requirements for publication in the Journal of Physiology.

Reviewer 1:

Thank you to the Reviewer for their systematic and insightful commentary on our manuscript. We have responded to the specific issues raised and have incorporated most suggestions in a revised manuscript.

1.1a. While this study may be the first to evaluate the relation of fibrosis to electrical remodeling in this animal model of hypertensive heart disease, fibrosis and arrhythmia risk has been evaluated in Langendorff-perfused and in vivo human hearts in myocardial infarction and nonischemic cardiomyopathy and some examples should be cited.

Many aspects of the structural and electrical remodelling linked here with arrhythmic risk in HHD also apply to other cardiomyopathies and we now discuss the role of tortuous conduction and arrhythmic risk from the seminal work of Professor de Bakker in lines 67-71 of the Introduction to our manuscript and expand further in lines 461-465, Section 4.3 of the Discussion (de Bakker et al, *JACC*, 15(7), 1594, 1990; de Bakker et al, *Circulation*, 98, 915, 1993; de Jong et al, *J. Cardiovasc. Pharmacol.* 57, 630, 2011). We give a more comprehensive outline of mechanisms by which fibrosis could contribute to arrhythmia risk and consider overlap between our results and comparable findings in human hearts with myocardial infarction and nonischemic cardiomyopathy and animal disease models. We also reference our own review on cardiac arrhythmogenic mechanisms at the tissue scale (Smaill et al, *Circ Res*, 112, 834, 2013) and a comprehensive review of mechanisms, including fibrosis, contributing to early afterdepolarisations and VT/VF (Karagueuzian et al, Front Physiol, 4(19), 1, 2013). These additions occur in lines 67-73 of the Introduction and lines 470-473 of the Discussion section 4.3.

We recognize that the final sentence of our original Introduction was broad, and we have elected to reword it (lines 91-93 Introduction) to say that our multi-modal electrophysiology and imaging approach provides a new multi-faceted view of VT/VF mechanisms in HHD.

1.1b. As discussed in de Bakker et al. 1990, the concept of regional complexity of fibrosis rather than estimates of fibrosis density as better predicting VT/VF risk has been raised in myocardial infarction (i.e. surviving myocytes near the infarcted region and "zigzag course of propagation') and should also be discussed in relation to the finding regarding the tortuous fibrosis described in the current study.

Like Professor de Bakker we are convinced that the regional complexity of replacement fibrosis plays a key role in arrhythmogenesis (Engelman et al, *Circ Arrhy Electrophys*, 3, 195, 2010; Smaill et al, *Circ Res*, 112, 834, 2013) and the results of this study are consistent with that view. In work we published in 2019 (Trew et al, *AJP Heart Circ*, 317(4), 743, 2019) we provided a companion to de Bakker et al (*JACC*, 15(7), 1594, 1990), showing direct measurements of tortuous (zigzag) activation through myocardial infarct border zone tissue strands in sheep hearts. We have referenced these works in several places in our revised manuscript. We highlight the complexity of fibrosis and the work of de Bakker et al (1990) on lines 461-463, Discussion section 4.3.

1.2. While connected myocytes were assessed based on proximity, the reason why Cx43 expression was not more extensively assessed should be provided. While Figure 4C details the comparison of connected adjacent cells to fibrosis density, the representative images do not clear provide examples of which adjacent myocytes would be considered connected in the images in Figure 4.

Individual myocytes were segmented in 3D (see also response to 1.6) as shown in Figure 4B and identified as linked to adjacent cells when the separation distance was $\leq$1 voxel (~0.4 μm) apart. Such connections were associated with discrete end-to-end, end-to-side or side-to-side junctions. While a Connexin 43 antibody was applied during the tissue block preparation, it did not penetrate consistently with passive diffusion and the resulting image channel did not provide useful quantitative data. It was only possible to make the descriptive statement (lines 387-389) that there was “some quantitative evidence of Cx43 plaques co-located with points of contact between myocytes.” We have now outlined the Cx43 labelling step in the revised manuscript (lines 165-166, Methods section 2.4) and have noted its quantitative limitations, supported by a recent study from our group (Sands et al. *Prog. Biophys. Mol. Biol.*, 168, 18-32, 2022) (lines 196-198, Methods section 2.4).

1.3. Could you provide further clarification on how fibrosis was identified on immunohistochemistry. Why was WGA used as cell membranes are also identified?

We used WGA as it diffuses readily through relatively thick tissue specimens (Sands et al. *Prog. Biophys. Mol. Biol.*, 168, 18-32, 2022). It is correct that this lectin binds to cell membranes and extracellular matrix in the myocardium and the coronary circulation. Picro-sirius red (PSR) is the standard histochemical stain used to quantify fibrosis, but it is not compatible with tissue clearing or antibody labels. Emde et al, (*Eur. J. Histochem*. 58, 2448, 2014) have shown that WGA performs as well as PSR in identifying fibrosis. In our study, the distributed fluorescence associated with cell membranes had no impact on the macroscale segmentation of replacement fibrosis but enabled us to segment cells in the detailed 3D images. In the revised version of the manuscript, we make these

points more directly, highlighting that WGA has been used previously to quantify myocardial fibrosis (lines 164-166, Methods section 2.4, line 375, Discussion section 4.1).

1.4. As VT/VF was inducible, were any reentrant circuits of VT able to be mapped to structure?

No. In this study, optical mapping was confined to the intact epicardial surface of the LV free wall and affected by electrical activity in the region adjacent to it. On the other hand, we have shown in Figure 3B that aggregates of replacement fibrosis most likely to provide a substrate for re-entry are located toward the mid and sub-endocardial layers of the LV. We identified VT from recorded ventricular electrograms rather than the optical maps. However, the epicardial maps exhibited rate-dependent instability (APD heterogeneity, beat-to-beat activation time and amplitude alternans - see Supporting Arrhythmia and Alternans Data: https://doi.org/10.17608/k6.auckland.16840138) that increased as VT was approached.

1.5. Would refrain from using the phrase "trend was marginally non-significant" and simply that it was non-significant.

Thank you. We have altered this in lines 299-303, Results section 3.3.

1.6. For the values in Table 1, can more details be provided about how the tissues were analyzed?

We have added further details to lines 200-205, Methods section 2.4. As many intact cells (i.e. those with a cell body that did not intersect the image boundary) as possible were semi-manually segmented from each 3D confocal image block in cleared tissue. Each segmented cell was analyzed by finding the eigenvectors of the covariance matrix for the segmented cell mask, with the vector corresponding to the largest eigenvalue providing the cell long axis direction and measure. At the center of mass, the cross-sectional area of the cell orthogonal to the long axis was computed by a digital resection of the segmented cell on that plane. The cell volume was found from the total volume of the segmentation (to the voxel resolution of the images).

1.7. For Figure 3A, could you clarify why data were binned from 15-165 degrees rather than showing the full 360-degree circumference?

We chose to consider a single sweep from the mid-septum to the LV free wall as it gave us more data for assessing statistics in each angle bin. Furthermore, in some early short-axis slices, the orientation of the upper surface was uncertain, and the anterior/posterior sides of the LV could not be differentiated. Our approach still gives a unique view of the circumferential distribution of fibrosis.

1.8. Figure 5: were similarly sized areas and locations assessed in the optical maps for each heart? The images in Figure 5A look somewhat disparate with only a scale bar for time. A scale bar for distance should also be provided.

A scale bar has been added to the images in figures 5 and 6, and each heart image has been confirmed as being consistent with the scale bar in that figure.

1.9. While non-hypertensive controls have been previously described as noted in the limitation, a discussion should be provided of how the current study compares to those historical controls. In particular, can insights be drawn on effects of age vs hypertension on the electrical remodeling described?

We have extended our discussion of several previous studies that show the differences in structure, electrical properties and VT/VF risk of SHR vs normotensive Wistar-Kyoto (WKY) rats from 6 months onward. For example, Herrmann et al (*Euro Heart J*, 16, 243, 1995) compared replacement scar in SHR vs normotensive rats and found more focal lesions in age matched SHR, concluding that "[a]ging only induces an increase in the total interstitial tissue" whereas the focal lesions are functions of hypertension and linked to microvascular alterations. All relevant studies we are aware of are referenced in the revised manuscript on lines 85-88 Introduction, lines 361-363, 366-373 Discussion section 4.1, lines 446-448, 452-455 Discussion section 4.3 and lines 501-509, Discussion section 4.5.

In response to this and other reviewer comments, we have performed a correlation and factor analysis for 10 electrophysiological and structural variables collected across the SHR animals. This is described in lines 215-228, Methods section 2.6, lines 322-344, Results section 3.4 and lines 393-396, 417-418, 433-436, 455-457 Discussion. This new analysis combined with data from previous studies utilising SHR and WKY rats, and figures 3, 5-8 have led us to conclude that age is not a strong predictor of VT/VF risk, whereas other variables associated with the development of HHD are better predictors.

Reviewer 2:

Preamble. These experiments are not innovative and have been routinely done by others but are now done with a focus on SHR hearts.

We do not claim that all components of this study (optical electrical mapping, directly electrical recording, tissue optical clearing, macroscopic short axis imaging and high-resolution 3D confocal imaging) are in themselves innovative. Indeed, we and others (Herrmann et al, *Euro Heart J*, 1995;**16,**243, Chan et al, *J Cardiovasc Pharmacol*, 2011;**57**:469; LeGrice et al, *AJP Heart Circ*, 2012;**303:**1353) have used some of these methods previously to characterise the electrical and structural remodelling that occurs at different time points in rats.

However, the 3D confocal imaging of optically cleared left ventricular tissue is not routine, although it is an emerging field of interest in the community and an area of research in which we are very active (Ashton et al, *Heart Rhythm*, 2019;16(5S):524; Sands et al, *Heart Rhythm*, 2020;17(5S):592; Sands et al, *Prog Biophys Mol Biol*, 2022;168:18). We maintain that the combination of cleared tissue, confocal imaging and cell segmentation from within the LV free wall and the parallel use of widefield/macroscopic imaging is innovative (lines 396-397, Discussion section 4.1). This has enabled us to identify structural indices of disease progression in a well-established animal model of hypertensive heart disease.

We also cannot find evidence of comparable unique data from other experiments. For example, our approach to feeding an LV free wall sub-epicardial pacing electrode from the endocardium (lines 119-120, Results section 2.2) allowed focal pacing without the optical map being obscured. The resulting spread of activation enabled us to categorize both longitudinal and transverse conduction velocity and we found that HHD progressing beyond 6 months leads to increasingly anisotropic conduction velocity as the diastolic interval decreases. We are not aware of other studies which have shown this in progressive HHD. Similarly, our finding that APD dispersion is similar for 12/18 month animals but increases non-linearly at low diastolic intervals is unique. As we have noted

already, we are not aware of other studies that have been able to optically clear ventricular tissue, label cell membranes and non-destructively image and segment cells and their architecture in 3D. In our opinion describing cells, their 3D morphology and likely coupling within intact tissue represents a novel technological advance.

2.1. The notion that fibrosis, be it on the surface, interstitial or patchy, can account for VT/VF risk has yet to be proven and the present data proposes a guilt by association. This is an on-going controversy and what is needed is data that relates the 'local' fibrosis to local electrical abnormalities.

There is substantial and widely accepted evidence linking the tissue conditions that promote reentrant activity to fibrosis in all chambers of the heart (de Bakker et al, *Circulation*, 1993;88:915 (800+ citations); Tanaka et al, *Circ Res*, 2007;101(8):839 (330+ citations)). This is from a mechanistic perspective and as a clinical biomarker exposed through, for example, late gadolinium delayed enhancement MRI (Schmidt et al, *Circulation*, 2007;115(15):2006). Mechanisms linked to fibrosis include tortuous conduction or activation slowing and block (de Bakker et al, *Circulation*, 1993;88:915), myocyte-myofibroblast interaction altering electrical loading (Miragoli et al,.***Circ Res***. 2006;**98:**801) and afterdepolarisation-mediated arrhythmia (Karagueuzian et al., *Front Physiol.* 2013;**4:**19), among others.

We have used well-established statistical methods to establish relationships between variables from individual animals that are highly significant. We think this goes beyond “guilt by association.” Furthermore, these relationships provide insights to the mechanisms involved and are consistent with the view that tortuous conduction and associated repolarisation heterogeneity make an important contribution to the arrhythmic substrate in this case.

We agree that relating local structure to local electrical function is ideal. Previously we have mapped intramural 3D propagation in and around scar of healed myocardial infarcts in a large animal experimental model (Trew et al, *AJP Heart Circ*, 2019;**317:**H743). Linked with tissue imaging this provided direct evidence of local mechanisms of delay and block and local tortuous pathways of viable myocardium. However, those data while gathered at higher 3D resolution than other studies were still unable to fully describe local abnormalities. Higher resolution signal mapping would interfere with electrical function, so this remains an open problem of bridging the scale gaps between mapping electrical function and structure and has been recognised before (de Bakker et al, *JACC*, 1990;15(7):1594). In our current study we have addressed these challenges by correlating variables collected at multiple scales using four independent measurements. In other studies, we used modelling techniques to bridge the scale gaps (Trew et al, *AJP Heart Circ*, 2019;**317:**H743).

2.2. The study ignores a great deal of data that relate electrical dysfunction in SHR rats to changes in ion channel expression, including Na+ channels, L-type Ca2+, lateralization of gap-junctions etc.

We carefully considered the published data regarding ion channel expression, intracellular calcium handling and gap junction distribution in the SHR. There are relatively few studies collecting data across a range of electrophysiological variables as HHD progresses over time. We highlighted the relevant studies on electrical remodelling in SHRs as HHD progresses in the Introduction (lines 85-88) (Tribulova et al, *Gen Physiol Biophys*, 22, 369, 2003; Nguyen et al, *J Physiol*, 594(6), 1689, 2016; Sung et al, *ESC Heart Failure*, ehf2.13013, 2020; Chan et al, *J Cardiovasc Pharmacol*, 57, 469, 2011). The contribution of sodium channels to reduced CV in progressive HHD (lines 418-421, Discussion section 4.2) was discussed as was the role of potassium channel down-regulation to prolongation of APD (lines 429-432, Discussion section 4.2).

2.3. There are no controls with aged matched non-SHR rats which may also develop patchy fibrosis as in the aged population.
2.6. Despite their claims, the authors do not demonstrate that arrhythmia risk is more reliably predicted by measures reflecting the extent of regional patchiness because they do not show controls where non-patchy fibrosis exhibits diminished or equal VT/VF risk.

We jointly address these two statements as we feel the important issues they raise can be effectively discussed together.

We designed this as a cross-sectional study in which 12 and 18 month-old SHRs were controlled with respect to a 6 month cohort with the same genetic traits. Aggregated fibrosis area (AFA) was low at 6 months, as was VT/VF risk. There were significant changes in these variables (and others) between 6 and 12/18 month cohorts, but no difference between the older groups (figures 3 and 7). These data indicated that age alone was not the primary determinant of structural remodelling or VT/VF risk, and this was confirmed with factor analysis (Figure 8). In contrast, VT/VF risk across the data set is predicted well by AFA and APD dispersion. Since these measures are related to the extent of LV cardiomyopathy, they directly reflect the progression of HHD in this animal model.

It is not obvious to us what normotensive age matched animals would add to this experimental design. We have been able to stratify the effects of age and disease progression, and demonstrate relationships between arrhythmic risk, structural remodelling and repolarisation heterogeneity that are highly significant.

The literature shows comprehensively that LV structure, electrical properties and VT/VF risk for SHR depart from normotensive Wistar-Kyoto (WKY) rats from about 6 months and remain on separate developmental paths. For example:

- By 6 months SHRs are already more susceptible to increased VT risk compared to controls (Tribulova et al, *Gen Physiol Biophys*, 2003;22:369; Nguyen et al, *J Physiol*, 2016;594(6):1689; Sung et al, *ESC Heart Failure*, 2020;ehf2.13013);
- APD durations in SHR vs normotensive rats separate at around 6 months and are significantly different from that time point on (Chan et al, *J Cardiovasc Pharmacol*, 2011;57:469 - 3, 6, 9, 12, 15, 18, 21, 24 months).
- There is considerable data showing increasing fibrosis with age in SHR vs WKY rats (LeGrice et al, *AJP Heart Circ*, 2012;303:1353 - ~ 3, 12, 18 and 24 months; Chan et al, *J Cardiovasc Pharmacol*, 2011;57:469 - 3, 6, 9, 12, 15, 18, 21, 24 months);
- There is data showing increased focal lesions or patchy replacement scar in SHR vs normotensive rats (in addition to the interstitial fibrosis consistent with aging) (Herrmann et al, *Euro Heart J*, 1995;16:243 - ~6, 12 and 18 months); and

We have discussed this in detail on lines 85-88 Introduction, lines 361-363, 366-373 Discussion section 4.1, lines 446-448, 452-455 Discussion section 4.3 and lines 501-509, Discussion section 4.5.

We recognize that our confocal imaging of optically cleared tissue is novel and that this has not been tested previously in normotensive controls. Therefore, we have studied post-mortem hearts from 18 month old WKY rats (n=4) and confirmed, using the same techniques as we used for SHR, that there is no evidence of patchy replacement fibrosis at that age. This is consistent with previous findings (Herrmann et al, *Euro Heart J*, 1995;16:243, LeGrice et al, *AJP Heart Circ*, 2012;303:1353). These new data are described in lines 175-180, Methods section 2.4, lines 244-246, Results section 3.1 and lines 372-373, Discussion section 4.1.

It is accepted that aged hearts display increased interstitial fibrosis, and they are more vulnerable to the induction of VT (de Jong et al, *Cardiovasc Pharmacol*, 2011;57(6):630). However, the evidence is

that focal replacement (patchy) fibrosis does not occur in normotensive rats at similar ages to SHR (Herrmann et al, *Euro Heart J*, 1995;16:243 - ~6, 12 and 18 months), and that study links this to pathological alterations in microvasculature unique to SHR. In a similar manner, Beliveau et al (*Comp Biol Med*, 2015;65:103) found using high-resolution MRI that normotensive rats developed diffuse fibrosis with age. We have discussed this more fully in lines 366-373, Discussion section 4.1.

2.4. There is no data on the nature and structure of the arrhythmias to support the notion that the changes in CV are localized to regions of high-patchiness and to sites where VF is initiated.

We do not believe we claimed that changes in CV were localized to regions of patchy fibrosis or linked to sites where VF is initiated. We have demonstrated that arrhythmic risk is strongly linked with measures of the distribution of replacement fibrosis in individual hearts, though not with age-related electrical remodelling. We have also found direct evidence of altered cell-to-cell coupling in regions of dense fibrosis, which would be consistent with increased CV anisotropy.

For reasons outlined in our response to 2.1, it is extremely challenging to make local measurements of electrical function in intramural regions where replacement fibrosis is concentrated. In large animal studies, we have previously recorded point-wise electrical activity from surviving myocardium within compact scar tissue, but CV cannot be determined within scar without further data (Trew et al, *AJP Heart Circ*, 2019;317:H743). In our revised manuscript we have expanded the discussion of CV anisotropy (lines 417-418, 421-427, Discussion section 4.2).

2.5a. The authors claim that there is increased arrhythmic risk with progression of hypertensive heart disease; this is not new.

We did not claim that increased arrhythmic risk with HHD progression was a new finding. There is strong published evidence linking HHD with increased arrhythmic risk and this was presented in the first paragraph of the Introduction as a motivation for our study. Furthermore, multiple studies have shown that VT/VF risk is substantially greater for SHRs than normotensive controls across the age range studied here (Tribulova et al, *Gen Physiol Biophys*. 2003;22:369; Nguyen et al, *J Physiol*. 2016;594:1689, 2016; Sung et al, *ESC Heart Failure*, 2020;ehf2.13013) and this is one of the main reasons that we opted for this animal model. Our claims are that:

(1) Arrhythmic risk is strongly predicted by the extent of aggregated replacement fibrosis in the LV. Replacement fibrosis proliferates with cardiomyopathy, providing a heart-specific measure of disease progression.

(2) Both risk and the structure measure are strongly associated with an independent measure of rate-dependent repolarization heterogeneity.

(3) Proliferation of fibrosis with progression of HHD increases the anisotropy of 3D cell coupling within affected regions.

(4) In this animal model, age was not a strong predictor of arrhythmic risk.

To the best of our knowledge, these findings have not presented before.

2.5b. Computational studies attempted to quantitatively relate arrhythmia risk to fibrosis, but thus far failed strictly by considering morphological changes of the tissue. This implies the need to include other changes that occur in HHD to explain their greater propensity to VT/VF.

In this manuscript, we refer to two well-cited papers from our group which use computer models constructed from high-resolution images of altered myocyte organisation due to replacement fibrosis (Engelman et al, *Circ Arrhythm Electrophys* 2010;**3:**195, Rutherford et al, *Circ Res* 2012;**111:**301). These show that morphologic changes are **sufficient** to generate reentry, due to

rate-dependent conduction slowing and block, even while other parameters are homogenous and constant. We are not alone in this finding, with the group of Prof Panfilov giving good examples of other studies (Kazbanov et al, *Scientific Reports* 2016; **6**(1):1; Pashakhanloo & Panfilov, *Physical Review Letters* 2021; **127**:098101; among many others).

However, while morphological changes may be **sufficient** to increase VT/VF risk they are not **necessary**, and other factors such as electrical remodelling, physical and chemical perturbation of the local extracellular context, altered inputs from the cardio-neural plexus, etc also alter VT/VF risk in HHD. In our original manuscript describing this wholly experimental study we linked VT/VF risk to both structural and electrophysiological remodelling. In the revised manuscript we have strengthened these links and investigated others by using multi-variable correlations and factor analysis across 10 variables collected from our four independent measurement techniques.

2.7. Late gadolinium-enhanced cardiac MRI might not have the spatial resolution to detect complex regional patchy fibrosis to diagnose/predict HHD arrhythmias.

We concur that resolution, segmentation error and partial volume errors all present challenges in accurate identification of replacement fibrosis with late gadolinium-enhanced MRI. However, as MRI sequences are tuned and new data modeling tools are developed (e.g. Zabihollahy et al, *Curr Cardiol Rep*. 2020;22:65), the expectation is that it will become more likely that patchy fibrosis can be reliably identified. Furthermore, we speculate that indices that rely on the aggregation of fibrotic regions may be less sensitive to error.

2.8. Although not essential, it would have been interesting to verify for sex-differences in VT/VF risk and/or patchy fibrosis.

We agree and will include this in any future studies with SHRs. Consistent with most others (e.g. Pahor et al, *Hypertension*. 1991;18:148) we focused on males to avoid introducing an additional confounding factor. We note that in the work of Chan et al (*J Cardiovasc Pharmacol*, 57, 469, 2011) there are no significant inter-sex differences in either APD or LV collagen fractions from 3-24 months in SHR or normotensive WKY animals. We have commented on our use of male animals in lines 102-103, Methods section 2.2.

Dear Dr Trew,

Re: JP-RP-2022-282526X "Arrhythmic risk in animal model of hypertensive heart disease predicted by distribution of patchy fibrosis" by Prashanna Khwaounjoo, Gregory Sands, Ian LeGrice, Girish Singh Ramlugun, Jesse Louis Ashton, Johanna Montgomery, Anne M Gillis, Bruce Smaill, and Mark Trew

Thank you for resubmitting your revised Research Article to The Journal of Physiology. It has been assessed by the original Reviewing Editor and Referees and has been well received. Some final revisions have been requested.

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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I hope you will find the comments helpful and have no difficulty returning your revisions within 2 weeks.

Your revised manuscript should be submitted online using the links in Author Tasks Link Not Available.

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REVISION CHECKLIST:

- Article file, including any tables and figure legends, must be in an editable format (eg Word)

- Abstract figure file (see above)

- Statistical Summary Document

- Upload each figure as a separate high quality file

- Upload a full Response to Referees, including a response to any Senior and Reviewing Editor Comments;

- Upload a copy of the manuscript with the changes highlighted.

You may also upload:

- A potential 'Cover Art' file for consideration as the Issue's cover image;

- Appropriate Supporting Information (Video, audio or data set https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#supp).

To create your 'Response to Referees' copy all the reports, including any comments from the Senior and Reviewing Editors, into a Word, or similar, file and respond to each point in colour or CAPITALS and upload this when you submit your revision.

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,

Harold D Schultz
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REQUIRED ITEMS:

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-Papers must comply with the Statistics Policy https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics

In summary:

-If n â‰¤ 30, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (preferably with data points included) are acceptable formats.

-If n > 30, then the entire raw dataset must be made available either as supporting information, or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

-'n' clearly defined (e.g. x cells from y slices in z animals) in the Methods. Authors should be mindful of pseudoreplication.

-All relevant 'n' values must be clearly stated in the main text, figures and tables, and the Statistical Summary Document (required upon revision)

-The most appropriate summary statistic (e.g. mean or median and standard deviation) must be used. Standard Error of the Mean (SEM) alone is not permitted.

-Exact p values must be stated. Authors must not use 'greater than' or 'less than'. Exact p values must be stated to three significant figures even when 'no statistical significance' is claimed.

-Statistics Summary Document completed appropriately upon revision

-Please include an Abstract Figure. The Abstract Figure is a piece of artwork designed to give readers an immediate understanding of the research and should summarise the main conclusions. If possible, the image should be easily 'readable' from left to right or top to bottom. It should show the physiological relevance of the manuscript so readers can assess the importance and content of its findings. Abstract Figures should not merely recapitulate other figures in the manuscript. Please try to keep the diagram as simple as possible and without superfluous information that may distract from the main conclusion(s). Abstract Figures must be provided by authors no later than the revised manuscript stage and should be uploaded as a separate file during online submission labelled as File Type 'Abstract Figure'. Please ensure that you include the figure legend in the main article file. All Abstract Figures should be created using BioRender. Authors should use The Journal's premium BioRender account to export high-resolution images. Details on how to use and access the premium account are included as part of this email.

EDITOR COMMENTS

Reviewing Editor:

Methods Details:
Expand arrhythmia index description in the methods

Comments for Authors to ensure the paper complies with the Statistics Policy:
Please provide all data points in graphs, not only summary data.

If the Statistical Summary Document has errors please describe what is incorrect?:
The statistical document needs to be updated to reflect the new figure 8, and any additional figures in the next revision.

Please provide file as Excel, not PDF

Comments to the Author:
The authors have substantially improved the MS. However, significant concerns remain that can be addressed without new data. Please provide a rebuttal to reviewer 2 comments.

In the current version of the MS, there is too much focus on the age dependence. Given that this is a HTN disease model, natural aging was NOT examined here. Animal age is simple a reflection of disease progression. Whether there is any relevance to natural aging is not known, and anyhow not the focus of the MS. Please tone down and remove any claims about aging from the MS. Given that no differences were found between 12 and 18 months old SHR rats, you could consider combining the dataset, especially since they seem underpowered to examine the various comparisons.

Rather, the authors should restructure and focus the MS on the correlation between tissue fibrosis and various EP parameters. This will also better emphasize the novel aspect of the work. The authors have quantified tissue fibrosis, CV APD and APD dispersion for each heart. A responsive revision should focus on analyzing APD, CV, CVI, APD dispersion and VTVF as a function of tissue fibrosis rather than a function of age, ideally with multiple new figures. Right now this information is only provided in Figure 8.

Since the authors did not measure cellular electrophysiology in each heart, it should be clearly stated that tissue fibrosis could be a marker for the degree of cellular EP remodeling, which could also explain the observed association with arrhythmia risk.

The title is misleading. Arrhythmia risk is NOT predicted by, but rather associated with the degree of tissue fibrosis. (nor by distribution of fibrosis). Please rephrase. Maybe "Arrhythmic risk is associated with the degree of patchy fibrosis in the left ventricle of SHR rats"

Please provide a more detailed description how the VTVF index was calculated. Fig. 1B needs a better explanation in the legend.

Abstract last sentence: The authors do not provide any evidence for tortious conduction. That sentence is conjecture and should be removed from the MS. Whether it occurs in the small rat heart and in this particular model remains to be determined. Unless you can provide experimental evidence for this mechanism, the language should be removed from the MS

The term replacement fibrosis seems inappropriate here, as indicated by reviewer 1. Unless you have specific evidence that myocytes are being replaced by fibrocytes, please rephrase.

The figures could be simplified as suggested below. This will allow more room for figures illustrating the correlation between tissue fibrosis and various EP parameters:

Figure 1 should be deleted and the panels incorporated into the results figures. Fig. 1A and C could go as insets in the

optical mapping results. Fig. 1B should go to the arrhythmia risk results figure, with a more informative legend. Fig. 1D to the CV results figure.

Figures 2 and 3 could be combined.

Figure 4: The quality of the 12 month and 18 month staining seems vastly different. Please clarify

Insert new figures comparing the relationship between tissue fibrosis and APD, APD dispersion, CVt, CVl, CV anisotropy, and any other parameters from Fig 8 you feel are relevant.

You could consider adding an ROC analysis of the various parameters to assess which is the best predictor of VTVF risk.

IMPORTANT: All online data have to be included in the MS. Please do not use hyperlinks to material on the authors server.

Senior Editor:

Comments for Authors to ensure the paper complies with the Statistics Policy:
Please include sample size in all figure legends. Please define samples, and if 'n' represents multiple determinations within animals, please state as x samples in x animals. Ensure the statistical test is stated when making statements on p values.

If the statistical summary document has errors please describe what is incorrect. :
Please include all statistical comparisons in the statistical summary document and submit as the excel file.

Comments to the Author:

The Title, Key Points, and Abstract need to be revised to better reflect the interpretation of the study.

Avoid statements of primacy to avoid controversy on who was first. It is better to state as 'novel'.

In the Methods section Ethical Approval, please include a statement that the investigators understand the ethical principles under which the journal operates and that their work complies with the animal ethics checklist as outlined in the journal policy: (please review: https://physoc.onlinelibrary.wiley.com/hub/animal-experiments)

Please include all statistical comparisons in the statistical summary document and submit as the Excel file.

REFEREE COMMENTS

Referee #1:

No additional comments, the revision is very responsive to the reviews.

Referee #2:

Specific Points:

1) SHRs develop considerable ion channel remodeling between 9 and 12 months: reduced INa, Ica,L, lateralization of Cx43 and reduced CV. The authors claim that there is no significant changes from measurements of AP upstroke from isolated

cells and from the Cerbai et al paper from 1994. These data have now been supplemented by newer studies. Hence VF/VT risk may well be predicted by the electrophysiological remodeling.

2) Normal healthy aging also produces changes in ion channel and electrophysiology with data coming from F-344 rat strain which has been adopted for aging studies because of their properties of exhibiting 'linear aging'. SHRs exhibit similar aging properties but at an earlier age than F-344 rats. The restitution kinetics of the CV shows that in aging CV decreases particularly at fast heart rates and upstroke velocity is normal at slow rates.

3) Line 236-239: Why is there no statistically significant data on wall thickness as a function of age? This is a concern because wall thickness must increase with age and weight of the heart unless the paraformaldehyde procedure alters wall thickness and the measurements of other important parameters.

4) Line 241: Fibrosis doesn't proliferate! It is important to be accurate with technical words. If fibrosis occupies a larger volume of the ECM and explanation must be provided as a function of myofibroblast expansion.

5) Lines 246-247: The term "replacement fibrosis" has been used in the literature as fibrosis built up to replace the region/ of myocardium depleted of live myocytes as in an MI. The data offered by the authors describe the increase in ECM 'area' or 'length' (AFA and AFL) which could be due to the cardiac hypertrophy, at age 6 and 9 months but perhaps some cell death at 18 months. In SHR ventricles, there is hypertrophy and an increase in fibrosis but is not necessarily "replacement fibrosis". If the authors wish to redefine the definition of this term and should explain what they mean by replacement fibrosis in SHR ventricles.

6) Line 248: "although there was considerable scatter in both cohorts (12 vs. 18 months) and no significant difference ($p = 0.5028$) between them. Corresponding analysis for normotensive WKY rats showed no evidence of replacement fibrosis in the left ventricle. This sentence should be clarified. Ae the authors arguing that there are no significant differences in fibrosis between 12 and 18 months old SHRs (bad scatter in the data) and that this is justified/consistent because the same thing happens with normotensive WKS rats? Another explanation is that WGA binds to N-acetyl-D-glucosamine and Sialic acid and may not be the best way to analyze fibrosis as the scatter of the data made it difficult to provide interpretable data. Perhaps there is a reason why Picrosirius red is used preferentially.

7) Delete Fig. 1B, it doesn't seem to be actual data.

8) There is no data on the initiation and maintenance of VF/VT and its relation to patchy fibrosis. Raw data showing APs, burst pacing and the structure of VF initiation. Is it localized near zones of patchy fibrosis?

END OF COMMENTS

1st Confidential Review **16-Mar-2022**

Khwaounjoo et al., propose that VT/VF risk in SHR hearts can be predicted by the **distribution of patchy fibrosis.** The authors use a multi-modal electrophysiology and imaging approach to investigate VT/VF mechanisms in HHD. Their main findings are that risk is predicted by two left ventricular measures: fibrosis quantity and structure, and epicardial APD dispersion ad they further propose that age, alone is not a good predictor of risk. The significance of the study is that a clear link between arrhythmic risk and fibrotic architectures in HHD will aid in the interpretation of late gadolinium-enhanced cardiac MRI and electrical mapping signals. The study uses a voltage sensitive dye to measure VT/VF risk following burst pacing and paraformaldehyde to clarify the tissues and measure fibrosis with WGA. Proximity measurements are used to evaluate the formation of gap-junction plaques and cell-cell coupling.

It seems that the goal(s) of the study are unclear and escapes logic. There is little doubt that fibrosis is an important predictor of arrhythmias and there are no claims in the literature that age alone is a predictor of arrhythmias. In the human population of 75 years old or older, about 10% are likely to develop AF. Severity of fibrosis is also well accepted to increase with aging and fibrosis alone is not sufficient to predict arrhythmias. Yet, this study focuses on fibrosis and VF/VT and is highly focused on only two parameters, trying to link them at the exclusion of all other possibilities. The notion that patchy fibrosis may be more arrhythmogenic than surface fibrosis is not new but as in other studies there is no direct measurement of electrical properties at the sites of large AFA or AFL to determine the effects of these structural defects. Important controls are missing and technical concerns with the use of the clarifying protocol and WGA further detract from the study.

Major Concerns.

1) SHRs develop considerable ion channel remodeling between 9 and 12 months: reduced I_{Na}, $I_{ca,L}$, lateralization of Cx43 and reduced CV. The authors claim that there is no significant changes from measurements of AP upstroke from isolated cells and from the Cerbai et al paper from 1994. These data have now been supplemented by newer studies. Hence VF/VT risk may well be predicted by the electrophysiological remodeling.
2) Normal healthy aging also produces changes in ion channel and electrophysiology with data coming from F-344 rat strain which has been adopted for aging studies because of their properties of exhibiting ‘linear aging’. SHRs exhibit similar aging properties but at an earlier age than F-344 rats. The restitution kinetics of the CV shows that in aging CV decreases particularly at fast heart rates and upstroke velocity is normal at slow rates.
3) Line 236-239: Why is there no statistically significant data on wall thickness as a function of age? This is a concern because wall thickness must increase with age and weight of the heart unless the paraformaldehyde procedure alters wall thickness and the measurements of other important parameters.
4) Line 241: Fibrosis doesn't proliferate! It is important to be accurate with technical words. If fibrosis occupies a larger volume of the ECM and explanation must be provided as a function of myofibroblast expansion.
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7) Delete Fig. 1B, it doesn’t seem to be actual data.
8) There is no data on the initiation and maintenance of VF/VT and its relation to patchy fibrosis. Raw data showing APs, burst pacing and the structure of VF initiation. Is it localized near zones of patchy fibrosis?

We thank the Editors and Referees who have reviewed this manuscript for their guidance and valuable criticisms. We believe that the changes made to it during this process have substantially improved our report of this study and trust that it now meets the standard required for publication in the Journal of Physiology. Systematic responses to issued raised in relation to the previous revision are given below.

Required Items:

We have sought to ensure that the revised manuscript complies with the Statistics Policy. Specifically:

- n values for all data are provided in the main text and relevant figure and table legends as well as the Statistical Summary Document.
- All data points are plotted where n<30 and when n≥30 the entire raw data set is provided as supporting information with the manuscript and also hosted on Figshare.com.
- Appropriate summary statistics are used.
- We have given exact p values to 5dp and these are always sufficient to differentiate from the threshold for significance (0.05).
- We have uploaded an accurate and comprehensive Statistics Summary Document as an Excel file.

Please include an Abstract Figure. The Abstract Figure is a piece of artwork designed to give readers an immediate understanding of the research and should summarise the main conclusions. If possible, ….. . Details on how to use and access the premium account are included as part of this email.

An Abstract Figure created using BioRender has been uploaded with the revised manuscript.

Response to Senior Editor:

1. *The Title, Key Points, and Abstract need to be revised to better reflect the interpretation of the study.*
   We have done this - see lines 1, 21 and 23 and lines 33-52 in this revised manuscript.
2. *Avoid statements of primacy to avoid controversy on who was first. It is better to state as 'novel'.*
   We now say "Multi-scale structural measures like these at the cellular and tissue scales are novel." on lines 434-435 in this revision.
3. *In the Methods section Ethical Approval, please include a statement that the investigators understand the ethical principles under which the journal operates and that their work complies with the animal ethics checklist as outlined in the journal policy: (please review: https://physoc.onlinelibrary.wiley.com/hub/animal-experiments)*
   On lines 98-99 of this revised manuscript we now state that: "The authors understand the ethical principles of the Journal of Physiology and this work complies with their policies."

Response to Reviewing Editor:

Methods Details:

1. *Expand arrhythmia index description in the methods.*
   We have done so. See Lines 132-142 in the revised manuscript.
2. *.. ensure the paper complies with the Statistics Policy: Please provide all data points in graphs, not only summary data.*
   In the revised version we only present summary data in figures 3C, 4B-D and 5B-C. In Figure 3C there would have been 72 datum, in each of figures 4B-D there would have been 134 datum and in each of figures 5B-C there would have been 136 datum. We found our trial figures with all datum

present difficult to read, and thus opted for summary data in these figures. The full raw data sets are presented in the supporting information.

Comments to Author:

1. *Please provide a rebuttal to reviewer 2 comments.*
   See later.

2. *In the current version of the MS, there is too much focus on the age dependence. Given that this is a HTN disease model, natural aging was NOT examined here. Animal age is simple a reflection of disease progression. Whether there is any relevance to natural aging is not known, and anyhow not the focus of the MS. Please tone down and remove any claims about aging from the MS. Given that no differences were found between 12 and 18 months old SHR rats, you could consider combining the dataset, especially since they seem underpowered to examine the various comparisons*.

   Rather, the authors should restructure and focus the MS on the correlation between tissue fibrosis and various EP parameters. This will also better emphasize the novel aspect of the work. The authors have quantified tissue fibrosis, CV APD and APD dispersion for each heart. A responsive revision should focus on analyzing APD, CV, CVI, APD dispersion and VTVF as a function of tissue fibrosis rather than a function of age, ideally with multiple new figures. Right now this information is only provided in Figure 8.

   This was not a study of aging per se; however, the age of each cohort of animals was a study variable (the ages of each animal to the nearest month are recorded in the full raw data sets presented in the supporting information) with the expectation that hypertensive heart disease would progress as the animals became older. This is reinforced in the revised manuscript. CV, CV anisotropy, APD and APD dispersion were quantified for animal cohorts around 6, 12 and 18 months in our SHRs and although animal numbers are relatively small (n=5 for each age), the experimental/statistical design (stimulation at up to 10 base cycle lengths; 2-way of analysis of variance and *post hoc* Tukey tests) shows highly significant differences ($P \leq 0.0001$) between 6 and 12 months and also identifies changes in APD70 though not other electrophysiological measures between 12 and 18 months. Combining data from these two latter groups does not affect these outcomes.

   A key finding of this study is that while the association of sampling age and VT/VF risk proved weak, the correspondence of VT/VF risk with measures of aggregated fibrosis and rate-dependent APD dispersion were extremely strong ($R^2 > 0.7$, $P < 0.00025$) for all animals studied. The fact that these associations were replicated across completely independent experimental measurements reinforces the power of these findings. This is presented in our revised figures 6 and 7. We have altered wording through the revised manuscript to ensure that age is not referred to as a study driver but appears as the sampling age in the data analysis.

   In the revised Figure 7 we have explicitly plotted a selection of key inter-variable relationships that were previously only given as raw data in the supporting data. These show (a) strong correlative relationships that feature in the network data model summary of revised Figure 7F, (b) moderate correlations that do not reach the threshold for inclusion in the network data model summary, and (c) an example of no correlation (between APD and the AFA index). Including plots of these relationships further supports the network summary of Figure 7F.

3. *Since the authors did not measure cellular electrophysiology in each heart, it should be clearly stated that tissue fibrosis could be a marker for the degree of cellular EP remodeling, which could also explain the observed association with arrhythmia risk.*

We are not entirely sure how to interpret this comment. One interpretation of "tissue fibrosis could be a marker for the degree of cellular EP remodeling" is that the former might be due to the latter. While there is evidence that electrical remodelling which drives atrial fibrillation can lead to atrial structural remodelling and fibrosis, structural derangement is viewed as the main cause of electrical dysfunction in the early phases of hypertensive heart disease as far as we are aware (see for example Burchfield JS, Xie M, Hill JA. Pathological Ventricular Remodeling. *Circulation* 2013;**128:**388-400). Our revised manuscript is consistent with this interpretation. Previously, we had acknowledged that epicardial optical mapping data cannot be used to make direct mechanistic inferences about the relative effects of electrical and structural remodelling when the structural substrate involved is most extensive in the inner LV wall. We have now revised the Limitations Section to expand on these issues and the three final sentences (Lines 569-574) now read: "Our preparation has the advantage that key aspects of the 3D structural remodelling that contributes to these rhythms are preserved, but we note that the interactions. between intramural LV structural remodelling and electrical dysfunction are complex and incompletely understood. The patchy fibrosis identified here in the inner wall of the LV should therefore be viewed as marking a potential substrate for re-entrant electrical activation."

We have also added a short additional paragraph that outlines direct and indirect mechanisms by which fibrosis is thought to modify ventricular tissue electrical properties in structural heart disease. Lines 520-527 of the Discussion section now read: "These mechanisms relate to heterogenous electrical propagation and electrotonic coupling in myocardium due to altered connection between adjacent myocytes. However, it has also been argued that the proliferation of myofibroblasts in fibrosis modulates cell electrical properties directly by electrotonic loading between myocytes and non-active cardiac tissue cells and via release of paracrine mediators such as TGF-β from the fibroblasts (de Jong, 2011; Kohl, 2014). This is justified by myocyte-fibroblast co-culture studies (Miragoli, 2006), computer modelling (Maleckar, 2009) and experimental studies in mice (Rubart 2018), but the extent to which these factors contribute to electrical dysfunction in structural heart disease remains unclear."

4. *The title is misleading. Arrhythmia risk is NOT predicted by, but rather associated with the degree of tissue fibrosis. (nor by distribution of fibrosis). Please rephrase. Maybe "Arrhythmic risk is associated with the degree of patchy fibrosis in the left ventricle of SHR rats"*

   We have changed the title to: "Multimodal analysis of arrhythmic risk in the spontaneously hypertensive rat"

5. *Please provide a more detailed description how the VTVF index was calculated. Fig. 1B needs a better explanation in the legend.*

   We have expanded the description of the VT/VF risk index (Lines 132-142) and included the proportionality relationship that we used to assign the integer risk index order to combinations of stimulus amplitude and frequency. Following editorial suggestion, we have removed the methods Figure 1. Our additional text is:

   "Two second bursts of 2 ms pulse trains with increasing frequencies (×4) and amplitudes (×3) were applied until VT or VF (>15 sustained complexes on the electrogram following cessation of stimulation) was induced (Rossi et al., 2017; Yang et al., 2007). The total stimulus current was used to assign VT/VF risk index integer values from 12 (high risk, low total current) to 1 (low risk, high total current). The total stimulus current, I_S (mA), is proportional to tissue and stimulus parameters as:

$$I_S \propto \sigma(f) f V.$$

   Here, σ is the local tissue conductivity (mS/mm) and a function of stimulus frequency, f is the pulse train frequency (10, 20, 50, 100 Hz) and V is the pulse amplitude (3, 5, 7.5 V). The local tissue conductivity dependence on frequency was modelled using data from Gabriel et al (2009), as $\sigma \approx 10^{-1.2} f^{0.1}$ (mS/mm)."

6. *Abstract last sentence: The authors do not provide any evidence for tortious conduction. That sentence is conjecture and should be removed from the MS. Whether it occurs in the small rat heart and in this particular model remains to be determined. Unless you can provide experimental evidence for this mechanism, the language should be removed from the MS.*

   We acknowledge that we have not provided direct evidence for tortuous conduction in this study – in fact, it is arguable if direct evidence is technologically possible to obtain in the rat. Electrophysiological mapping data were acquired from the epicardial surface of the LV free wall, whereas the aggregated fibrosis associated with increased VT/VF risk is concentrated in the subendocardial LV wall. We have changed the final sentence of the abstract to: " The findings are consistent with the notion that VT/VF risk is associated with rate-dependent repolarization heterogeneity caused by structural remodelling and reduced lateral electrical coupling between LV myocytes as HHD progresses." We have also removed references to tortuous conduction in Lines 581 and 582 in the Conclusions to this revision of the manuscript.

   However, there is substantial experimental evidence across species cited in this manuscript which demonstrates that replacement fibrosis in structural heart disease gives rise to intramural tortuous regional conduction (see deBakker et al., 1990; de Bakker et al., 1993) and recently from our group (Trew et al., 2019), although this is not yet the case for direct modulation of cellular electrophysiological properties by fibroblasts (see 3 above). We believe that it is appropriate to consider both in the Discussion section.

7. *The term replacement fibrosis seems inappropriate here, as indicated by reviewer 1. Unless you have specific evidence that myocytes are being replaced by fibrocytes, please rephrase.*

   We have substituted the term replacement fibrosis with patchy fibrosis throughout the manuscript. The high-resolution imaging reported in this manuscript does provide clear evidence of replacement fibrosis (extensive aggregations of connective tissue with marked reductions in the extent of coupling between adjacent myocytes) at 12 months and 18 months (see Figure 3A and 3C). However, we acknowledge that more cell-scale data would be required to demonstrate a link between increased AFA and replacement fibrosis.

8. *The figures could be simplified as suggested below. This will allow more room for figures illustrating the correlation between tissue fibrosis and various EP parameters:*

   Figure 1 should be deleted and the panels incorporated into the results figures. Fig. 1A and C could go as insets in the optical mapping results. Fig. 1B should go to the arrhythmia risk results figure, with a more informative legend. Fig. 1D to the CV results figure.

   Following discussion among the authors, we have decided to remove the original Figure 1.
   However, we have opted not to put the constituent parts into results figures, as this would make them too complex. The methods are well described in the text. Should readers

   Figures 2 and 3 could be combined.

   We have left this as it is to preserve separation between Methods and Results.

   Figure 4: The quality of the 12 month and 18 month staining seems vastly different. Please clarify

   Our 3D high-resolution cell scale images of optically cleared tissue are novel in cardiac imaging. We do not believe that the quality of staining is materially different between the 12 and 18 month samples shown in the revised Figure 3. However, there are differences in how the images appear. Both the extent of autofluorescence (which increases the contrast between myocytes and extracellular space) and the orientation of the cell axis with respect to the imaging plane vary between these images. We now say on Lines 259-263 of the Results section that "Auto-fluorescence and orientation of the cell axes to the imaging plane varies across the image sets. This

alters the contrast between myocytes and extracellular space, while the point-spread function of the objective (Sands et al., 2022) is anisotropic with 2-3× better resolution in the imaging plane than transverse to it."

Insert new figures comparing the relationship between tissue fibrosis and APD, APD dispersion, CVt, CVl, CV anisotropy, and any other parameters from Fig 8 you feel are relevant.

We have done this. See Figure 7 in this revision of the manuscript.

9. *You could consider adding an ROC analysis of the various parameters to assess which is the best predictor of VTVF risk.*

   We have considered this but believe that this issue is well covered in the factor analysis illustrated in Figure 7.

10. *All online data have to be included in the MS. Please do not use hyperlinks to material on the author's server.*

    Where data have n < 30 these are included in the manuscript. Data for n > 30 they are included in supporting data placed on figshare with unique public DOI, e.g. doi.org/10.17608/k6.auckland.16840198, etc. We have removed hyperlinks from the manuscript but refer to the data set in the Supporting Information section which links to the figshare DOI.

Reponse to Referee #2:

1) *SHRs develop considerable ion channel remodeling between 9 and 12 months: reduced INa, Ica,L, lateralization of Cx43 and reduced CV. The authors claim that there is no significant changes from measurements of AP upstroke from isolated cells and from the Cerbai et al paper from 1994. These data have now been supplemented by newer studies. Hence VF/VT risk may well be predicted by the electrophysiological remodeling.*

   Altered I_{Na} and $I_{Ca,L}$ channel expression and other remodelling between 6 and 12 months may explain features of our experimental observations. However, we note that it is challenging to unpack the system level impact of this remodelling, and it can be unexpected. For example, it has been shown that CV and CV restitution remains relatively unaltered with Cx43 knockout of up to 60%. We discussed this in detail in a review of 3D impulse propagation: Smaill et al, *Circ Res*, 112:834, 2013. In our manuscript we directly summarise Cerbai et al. (1994) when we say they show: (1) AP kinetics in HHD modulated by altered calcium handling, (2) preserved sodium channel density as AP upstroke rate was consistent in isolated myocytes from 3 and 18 month SHRs, and (3) APD prolongation linked to down-regulated Ito. In multiple independent literature searches by the authors, we have been unable to find the newer studies referred to by the Referee and we would be pleased to receive appropriate citations from them.

   We have shown that electrophysiological remodelling does occur, particularly between 6 and 12 months, even though we have not been able to find recent supporting information on the cellular mechanisms that might explain this observation. However, our analysis also demonstrates that there is no clear association between either the reduced CV or the increase in APD reported and increased VT/VF risk.

   In our revised manuscript, we provide an expanded and up-to-date account of additional possible direct and indirect mechanisms contributing to altered electrical function and exacerbated arrhythmic risk. These additions are in lines 520-527 of the Discussion section.

2) *Normal healthy aging also produces changes in ion channel and electrophysiology with data coming from F-344 rat strain which has been adopted for aging studies because of their properties of exhibiting 'linear aging'. SHRs exhibit similar aging properties but at an earlier age than F-344 rats. The restitution kinetics of the CV shows that in aging CV decreases particularly at fast heart rates and upstroke velocity is normal at slow rates.*

   Our study was not designed to relate the progression of HHD in SHRs to normal aging, but it is not surprising that the above mentioned data suggest earlier development of features typically associated with aging in SHRs. In our discussion section 4.1 we say: "With aging, there is increased interstitial fibrosis but not patchy fibrosis in murine models (de Jong et al., 2011). At some time point between 6 and 12 months, the increase in interstitial fibrosis due to aging diverges between normotensive WKY rats and SHR (Herrmann et al., 1995). Unlike WKY rats, SHR increasingly develop patchy fibrosis from 12 months, and this tends to localize mainly near the endocardium (Herrmann et al., 1995; Pahor et al., 1991). Our findings from both SHR and WKY tissue imaging are consistent with these earlier studies."

3) *Line 236-239: Why is there no statistically significant data on wall thickness as a function of age? This is a concern because wall thickness must increase with age and weight of the heart unless the paraformaldehyde procedure alters wall thickness and the measurements of other important parameters.*

   We agree that by around 12 months SHR typically display increased wall thickness, myocyte cross section and fibrosis. Over the subsequent 12 months, wall thickness and myocyte cross section do not tend to increase further, but LV dilation, necrosis and replacement fibrosis do tend to increase. See for example: Engelmann et al, *Circ Res* 60:487, 1987, and Le Grice et al, *AJP Heart Circ Physiol* 303:H1353, 2012. We observed these same trends.

   Quantitatively, our wall thickness values are similar dimensions to SHR at similar ages in Engelmann et al (1987) and our cell cross sections are within the SHR ranges given by Le Grice et al (2021). This does not support the notion of systematic differences that could be linked to tissue processing. We also note that our structural measures (such as circumferential location, transmural proportion, fibrosis density and AFA index) are all normalised and therefore nominally independent of dimension.

4) *Line 241: Fibrosis doesn't proliferate! It is important to be accurate with technical words. If fibrosis occupies a larger volume of the ECM and explanation must be provided as a function of myofibroblast expansion.*

   We have replaced "proliferation" with "increased" in the revised manuscript.

5) *Lines 246-247: The term "replacement fibrosis" has been used in the literature as fibrosis built up to replace the region/ of myocardium depleted of live myocytes as in an MI. The data offered by the authors describe the increase in ECM 'area' or 'length' (AFA and AFL) which could be due to the cardiac hypertrophy, at age 6 and 9 months but perhaps some cell death at 18 months. In SHR ventricles, there is hypertrophy and an increase in fibrosis but is not necessarily "replacement fibrosis". If the authors wish to redefine the definition of this term and should explain what they mean by replacement fibrosis in SHR ventricles.*

   We acknowledge that the aggregations of fibrosis identified by AFA and AFL are not necessarily replacement fibrosis and we now refer to this as "patchy fibrosis" throughout the manuscript. High-resolution cell imaging (revised Figure 3) provides evidence of replacement fibrosis (extensive regions of connective tissue and reduced coupling between adjacent myocytes) at 12 months and

18 months. However, we acknowledge that more cell-scale data would be required to demonstrate a link between increased AFA and replacement fibrosis.

6) *Line 248: "although there was considerable scatter in both cohorts (12 vs. 18 months) and no significant difference (p = 0.5028) between them. Corresponding analysis for normotensive WKY rats showed no evidence of replacement fibrosis in the left ventricle. This sentence should be clarified. Are the authors arguing that there are no significant differences in fibrosis between 12 and 18 months old SHRs (bad scatter in the data) and that this is justified/consistent because the same thing happens with normotensive WKS rats? Another explanation is that WGA binds to N-acetyl-D-glucosamine and Sialic acid and may not be the best way to analyze fibrosis as the scatter of the data made it difficult to provide interpretable data. Perhaps there is a reason why Picrosirius red is used preferentially.*

We apologise for the lack of clarity, and on Lines 250-254 of the revised manuscript we now state: "Measures were significantly greater at 12 and 18 months compared to 6 months (6 vs 12, 6 vs 18 month, p = 0.0489, 0.0081 respectively, one-way ANOVA and posthoc Tukey), but there was considerable overlap between 12 and 18 month cohorts and no significant difference (p = 0.5028, one-way ANOVA and posthoc Tukey) between them. Imaging and analysis for 18 month normotensive WKY rats showed no evidence of patchy fibrosis in the left ventricle (Additional data are available at https://doi.org/10.17608/k6.auckland.16829017)."

In previous studies we have used picrosirius red (PSR) extensively to label collagen in the heart (e.g. Rutherford et al., 2012). It is more specific than WGA but cannot easily be combined with immuno-histochemical probes such as the Cx43 antibodies used here. However, Emden et al (2014) reported that WGA is as effective as PSR for quantifying myocardial fibrosis. Consistent with this, the results presented here show a very strong association between AFA determined from low resolution images of WGA-labelled LV short-axis specimens and the extent of fibrosis identified by cell-scale imaging in the same specimens (see response to 5 above).

7) *Delete Fig. 1B, it doesn't seem to be actual data.*

This was a method schematic showing the assignment of risk index to the combinations of burst pacing amplitude and frequency. However, we have deleted the figure in this revision of the manuscript and describe the risk index with combinations of pulse amplitude and frequency in terms of total stimulus current.

8) *There is no data on the initiation and maintenance of VF/VT and its relation to patchy fibrosis. Raw data showing APs, burst pacing and the structure of VF initiation. Is it localized near zones of patchy fibrosis?*

VT/VF incidence was determined from electrogram recordings and not optical maps. The optical maps were used to estimate epicardial/subepicardial electrical properties during focal pacing from within the field of view. These regional maps are difficult to interpret during the unstable global rhythms that characterise VT/VF and were therefore not recorded after this was induced. However, we did record maps during the cycles of concordant and discordant alternans rhythm that occurred as stimulus rate was increased immediately prior to the incidence of VT/VF (raw maps are provided in the supporting data). It is widely argued that these rhythms reflect electrical instability in regions where structure and/or electrical properties are heterogeneous. Unfortunately we are not able to map activity near zones of patchy fibrosis in these intact hearts, because they are concentrated in the inner LV wall. However, we have shown in previous *in-vivo* large animal studies that conduction is altered adjacent to patches of replacement fibrosis (Trew et al, *AJP Heart Circ Physiol* 317(4):H743, 2019) and that VT initiation and maintenance can be explicitly affected by local connective tissue structures (Trew et al, *Heart Rhythm* 10(5S):S58, 2013).

Dear Dr Trew,

Re: JP-RP-2022-282526XR1 "Multimodal analysis of arrhythmic risk in the spontaneously hypertensive rat" by Prashanna Khwaounjoo, Gregory Sands, Ian LeGrice, Girish Singh Ramlugun, Jesse Louis Ashton, Johanna Montgomery, Anne M Gillis, Bruce Smaill, and Mark Trew

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EDITOR COMMENTS

Reviewing Editor:

The revised MS has addressed almost all remaining concerns.

My only remaining concern is for the authors to write a more informative title that includes the major finding of the study. Informative titles increase the likelihood that the work will be recognized. Maybe "Multimodal imaging identifies fibrosis architecture and action potential dispersion as major predictors of arrhythmic risk in spontaneously hypertensive rats".

Senior Editor:

Although the study was well received in revision, we also want to ensure that it will receive deserved attention on search strategies. The title remains too ambiguous (as the senior editor pointed out previously). The reviewing editor has provided a good alternative example.

To differentiate description of results from statements of fact or conclusions, results should be stated in past tense of the verb, because they were obtained in the past. Abstract and Results section should describe results using the past tense of the verb, for e.g. line 41-42: "These (sic) showed increased fibrosis from 6 months... Myocyte cross-section increased at 12 months, while inter-myocyte connections were reduced markedly with fibrosis." etc. and throughout Results.

Methods descriptions also are always stated in past tense, because they were performed in the past, e.g. line 36: "Risk was electrically assessed in Langendorff-perfused... membrane voltage maps were acquired..." etc.

In Figures 2,4,5,6 the actual p values for the comparisons should be in the figure rather than a symbol.

REFEREE COMMENTS

Referee #1:

The revisions are appropriate and I congratulate the authors on a useful contribution to the field.

_____

END OF COMMENTS

2nd Confidential Review **16-Jul-2022**

Thank you to the Reviewing and Senior Editor for their support and comments and to Referee 1 for their support. We have made all the suggested alterations.

Reviewing Editor:

The revised MS has addressed almost all remaining concerns.

My only remaining concern is for the authors to write a more informative title that includes the major finding of the study. Informative titles increase the likelihood that the work will be recognized. Maybe "Multimodal imaging identifies fibrosis architecture and action potential dispersion as major predictors of arrhythmic risk in spontaneously hypertensive rats".

We have modified the title to be more descriptive and it is now aligned with the Reviewing Editor's suggestion: "Multimodal imaging shows fibrosis architecture and action potential dispersion are predictors of arrhythmic risk in spontaneous hypertensive rats". This remains under the 150 character title limit.

Senior Editor:

Although the study was well received in revision, we also want to ensure that it will receive deserved attention on search strategies. The title remains too ambiguous (as the senior editor pointed out previously). The reviewing editor has provided a good alternative example.

We have modified the title as above.

To differentiate description of results from statements of fact or conclusions, results should be stated in past tense of the verb, because they were obtained in the past. Abstract and Results section should describe results using the past tense of the verb, for e.g. line 41-42: "These (sic) showed increased fibrosis from 6 months... Myocyte cross-section increased at 12 months, while inter-myocyte connections were reduced markedly with fibrosis." etc. and throughout Results.

Methods descriptions also are always stated in past tense, because they were performed in the past, e.g. line 36: "Risk was electrically assessed in Langendorff-perfused... membrane voltage maps were acquired..." etc.

We have gone through the manuscript carefully to ensure that all Methods and Results presented in the Abstract are in past tense. We have confirmed that the Methods are also in the past tense. All stated Results are now in the past tense, unless referring directly to a figure and what it shows.

In Figures 2,4,5,6 the actual p values for the comparisons should be in the figure rather than a symbol.

We have altered these figures to include the p values.

Dear Dr Trew,

Re: JP-RP-2022-282526XR2 "Multimodal imaging shows fibrosis architecture and action potential dispersion are predictors of arrhythmic risk in spontaneous hypertensive rats" by Prashanna Khwaounjoo, Gregory Sands, Ian LeGrice, Girish Singh Ramlugun, Jesse Louis Ashton, Johanna Montgomery, Anne M Gillis, Bruce Smaill, and Mark L Trew

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3rd Confidential Review **03-Aug-2022**