**Peer-Review Record**

# Long-term efficacy and safety of cardiac genome editing for catecholaminergic polymorphic ventricular tachycardia

*J Cardiovasc Aging* 2024;4:8. https://www.oaepublish.com/articles/jca.2023.42

by Oliver M. Moore, Yuriana Aguilar-Sanchez, Satadru K. Lahiri, Mohit M. Hulsurkar, J. Alberto Navarro-Garcia, Tarah A. Word, Joshua A. Keefe, Dean Barazi, Elda M. Munivez, Charles T. Moore, Vaidya Parthasarathy, Jaysón Davidson, William R. Lagor, So Hyun Park, Gang Bao, Christina Y. Miyake, Xander H. T. Wehrens

**Received:** 22 Nov 2023 **| First Decision:** 14 Dec 2023 **| Revised:** 20 Dec 2023 **| Accepted:** 28 Dec 2023 **| Published:** 5 Jan 2024

**Academic Editor:** Ali J. Marian

**Reviewer 1: Anonymous**

**Reviewer 2: Anonymous**

## Round 1

### Reviewer 1 Report

An original research article describing the long-term efficacy of CRISPR-Cas9 genome editing in the RYR2 gene in a mouse model of CPVT by Moore et al.

Comments

The study complements the previous work of the group and provides data on the overall safety of CRISPR-Cas9-mediated genome editing. The data are comprehensive. The authors have presented their findings clearly. Overall, this reviewer has no major concerns with data presentation or interpretation. The reviewer suggests a few minor changes for clarity.

It would be informative if the authors could provide data on sustained expression of SaCas9 and gRNA in selected cell types, particularly cardiomyocytes. Said otherwise, were SaCas9 and gRNA transiently or persistently expressed in cardiomyocytes, and if the latter, how long did their expressions last? Were they expressed at 12 months after the AAV9 injection?

It would be informative to describe whether there were single base insertions or deletions in the AAV9-saCas9-gRNA treated group and whether point or frameshift mutations were detected in the gene-edited group (resulting from NHEJ). Likewise, the authors might wish to expand on the fate of mRNA species carrying indels (small) or large insertion/deletions

and even point mutations resulting from NHEJ in cardiomyocytes and whether any of these mRNA species were transcribed into aberrant RYR2 proteins. Data on the RYR2 protein is rather limited in the manuscript but perhaps understandable given the difficulty of working with a large protein.

Regarding the above point, the long-term efficacy of editing the Ryr2 gene is shown at the RNA level by RT-PCR. Data on the RYR2 protein level would be valuable.

Recognizing that neither AAV9 nor U6 affords cardiomyocyte-specificity, the authors might wish to indicate which other tissues and cell types also expressed gRNA and whether there were any immune reactions to Cas9 and gRNA.

Considering the above point, the reduction in the Ryr2 mRNA in the treatment group was ~ 45% and the protein level ~ 39%, whereas the mutant allele comprised only 32% of the DNA copies. Figure 1C shows that ~ 19% of the sequence reads contained the variant allele. The differences are likely within the variability of the technique. Nevertheless, it has caught this reviewer's attention that the authors do not observe much allelic compensation.

The reviewer also recognizes that off-target effects were analyzed by amplicon sequencing, which is specific to the intended target locus. The authors might wish to emphasize the limitations of their approach for the detection of off-target effects.

The reviewer suggests changing the annotation of the variant to p.Arg176Gln

The reviewer suggests specifying whether mRNA or gene is meant by "Ryr2" throughout the manuscript.

In the abstract and the text, the reviewer suggests describing the specific data rather than the percentage. It is best to state the actual numbers and include the percentages in parentheses. For example, in line 238, 71% of the R176Q/+ …. Better state X out of Y mice (71%).

Per the abstract phenotypic characterization was performed at 6 weeks after AAV9 injection to 10-day-old neonates, i.e., at ~ 8 weeks of age. The reviewer suggests indicating in the abstract that EP studies were also performed at an older age.

The reviewer suggests revising the following statement: "Unlike other disorders associated with ventricular arrhythmias, implantable cardioverter-defibrillators (ICDs) may increase the risk of sudden cardiac death in CPVT patients due to a higher burden of inappropriate shocks and vulnerability to complications, such as electric storm [3,4]". An ICD might be ineffective in certain subset of arrhythmias in patients with CPVT but the cited original article does not show an increased risk of sudden cardiac death because of ICDs. The second cited reference is a review article and does not contain original data. It would be more accurate – in this reviewer's opinion - to state that ICDs might be ineffective in a subset of patients with CPVT such as those with incessant ventricular arrhythmias.

Figure 1E. If the data is presented as fold change and normalized to Gapdh mRNA, the mean in the control group should be around 1 (instead of close to 1.5). It seems that the data is relative to RQ-con. It should be to wild type.

Figure 1F. The differences in the RYR2 protein levels are hard to discern. Would a large panel or a darker exposure show the differences better?

Regarding the presentation of ventricular arrhythmias, it would be more informative to describe additional characteristics besides the presence or absence of ventricular arrhythmias or the duration. The number of mice in these experiments should be depicted in the figures or stated in the text (whenever not described in the figure legend).

The duration of ventricular arrhythmias in the RQ-con seems to be very short (in both sets of experiments), despite pacing electric stimulation and treatment with ISO and caffeine, which casts some shadow on the model. The authors might wish to comment on the shortcomings of the model.

Regarding the data on calcium sparks, there is an issue of independence of the samples in each genotype, meaning that data obtained in several cardiomyocytes from each mouse should be considered as one unit and the mean number per mouse per genotype should be presented and used in data analysis. The reviewer recognizes that this form of presentation is common with these types of studies and there are plenty of studies that have used a similar form of data presentation and analysis. Nevertheless, the readers should be informed that it is incorrect to mix/pool data from multiple cardiomyocytes (20 cells here) obtained from multiple mice (5 or 6 mice) as one group. The approach violates the fundamental principle of independence of the samples mandated by the statistical methods used in these analyses. Likewise, these measurements are extremely soft phenotypes, which are subject to massive underrepresentation (few cells out of several million cells per heart), ascertainment bias, and selection bias and show massive dispersion. The reviewer leaves it to the discretion of the authors to present their data as is but the reviewer encourages the authors to rise above the common trend and either perform the proper statistical analysis that considers the dependence of the samples or present the data per mouse.

Line 379., The reviewer suggests changing very efficient to efficient.

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**Author Response**

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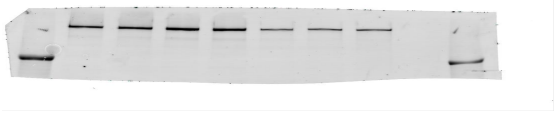
Thank you for this suggestion. We only had heart lysates available, but we do not consider this to be a limitation given that RyR2 is only expression within cardiomyocytes. New PCR experiments were performed to quantify the levels of AAV9 genome in the ventricles of R176Q/+ mice. Please note that these vectors do not undergo dilution since cardiomyocytes are not dividing (unlike for example hepatocytes). In addition, qRT-PCR was performed to measure SaCas9 mRNA levels. SaCas9 mRNA levels remained elevated in the hearts of R176Q/+ mice at 12-months after AAV9 administration. These new findings are included in the new Supplemental Figure 5.

It would be informative to describe whether there were single base insertions or deletions in the AAV9-saCas9-gRNA treated group and whether point or frameshift mutations were detected in the gene-edited group (resulting from NHEJ). Likewise, the authors might wish to expand on the fate of mRNA species carrying indels (small) or large insertion/deletions and even point mutations resulting from NHEJ in cardiomyocytes and whether any of these mRNA species were transcribed into aberrant RYR2 proteins. Data on the RYR2 protein is rather limited in the manuscript but perhaps understandable given the difficulty of working with a large protein.

We would like to point out that single base insertion and deletion were seen in the AAV9-saCas9-gRNA treated group as shown in Figure 1B. Analysis of the western blot gel of RyR2 between RQ and RQ-gRNA ventricles show similar band sizes with no indication of cleavage, although this does not definitively exclude the presence of some truncated RyR2 proteins.

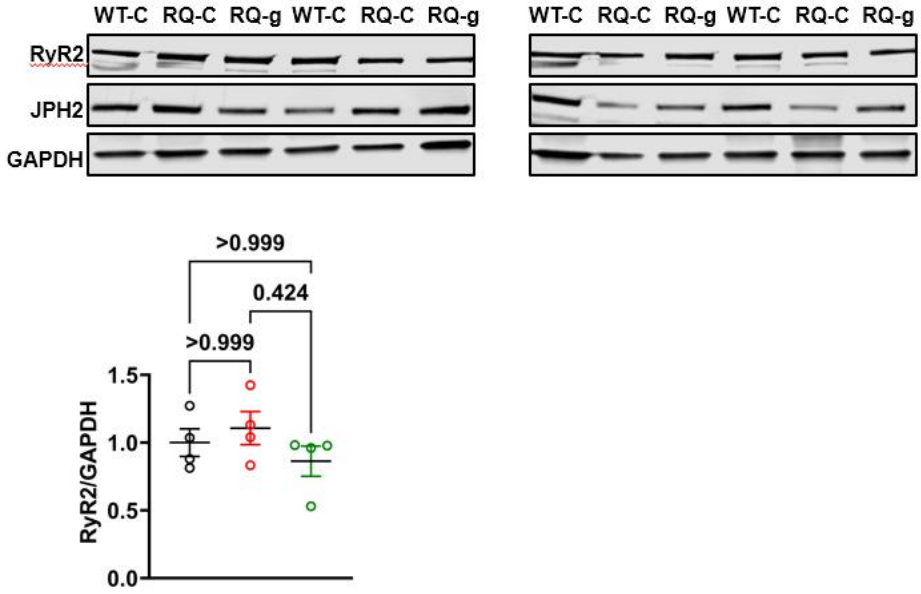
RYR2 RQ-C, RQ-C, RQ-C, RQ-C, RQ-g, RQ-g, RQ-g



GAPDH

Regarding the above point, the long-term efficacy of editing the Ryr2 gene is shown at the RNA level by RT-PCR. Data on the RYR2 protein level would be valuable.

We agree with the reviewer that it is important to also examine the protein levels. Unfortunately, we only had a few remaining samples to work with. The reviewer-only figure shows that there was a decrease in RyR2 protein levels, but the difference was not significant. However, we are reluctant to include this in the paper because these studies are underpowered and might be confusing.




Recognizing that neither AAV9 nor U6 affords cardiomyocyte-specificity, the authors might wish to indicate which other tissues and cell types also expressed gRNA and whether there were any immune reactions to Cas9 and gRNA.

We agree that specificity and immune reactions are important concerns for any therapy including genome editing. While the U6 promoter does not afford cardiomyocyte-specificity, we concurrently utilized the human cardiac troponin T promoter described in Werfel *Cardiovasc Res*. et al 2014 (PMID 25082846) to be cardiac specific. To verify if there were any immune reaction to Cas9 or gRNA, we did H&E staining of both liver and heart and saw no increase in infiltration of immune cells at least at 12 months post injection (Supplemental figure 4).

Considering the above point, the reduction in the Ryr2 mRNA in the treatment group was ~ 45% and the protein level ~ 39%, whereas the mutant allele comprised only 32% of the DNA copies. Figure 1C shows that ~ 19% of the sequence reads contained the variant allele. The differences are likely within the variability of the technique. Nevertheless, it has caught this reviewer's attention that the authors do not observe much allelic compensation.

We agree with the reviewer that there are limitations in NGS and that the lack of allelic compensation is interesting. The ~19% editing efficiency in Figure 1C is most likely an

underestimation of total editing due to our method of amplicon sequencing. First, the bulk tissue genomic sequencing includes fibroblasts and immune cell DNA that do not express SaCas9 either due to the tropism of AAV9 or the human cardiac troponin T promoter described in Werfel *Cardiovasc Res*. et al 2014 (PMID 25082846). Second, the amplicon size limits the reading frame. Thus, the NGS did not detect large insertions and deletions that extended. This is addressed in the discussion on page 18. Finally, the lack of allelic compensation while interesting is also expected, as similar decreases in RyR2 were seen in a genetic model of heterozygous RyR2 knockout (Zheng *J Mol Cell Cardiol*. 2022; PMID: 35413295).

The reviewer also recognizes that off-target effects were analyzed by amplicon sequencing, which is specific to the intended target locus. The authors might wish to emphasize the limitations of their approach for the detection of off-target effects.

We agree with the limitations in amplicon sequencing. We have added the following text to page 18.

*"Likewise, while we chose our guide to have no off targets with <3 mismatches in silico, our in vivo analysis of off target effects was limited to the amplicon region of the wild type allele."*

The reviewer suggests changing the annotation of the variant to p.Arg176Gln.

We have changed the initial annotation to p.Arg176Gln. We used R176Q in subsequent nomenclature since it is much easier to read as a shorthand of the variant.

The reviewer suggests specifying whether mRNA or gene is meant by "Ryr2" throughout the manuscript.

We have added mRNA or gene following RyR2 throughout the manuscript.

In the abstract and the text, the reviewer suggests describing the specific data rather than the percentage. It is best to state the actual numbers and include the percentages in parentheses. For example, in line 238, 71% of the R176Q/+ ⋯. Better state X out of Y mice (71%).

We thank the reviewer for the suggestion and have made the corresponding changes.

Per the abstract phenotypic characterization was performed at 6 weeks after AAV9 injection to 10-day-old neonates, i.e., at ~ 8 weeks of age. The reviewer suggests indicating in the abstract that EP studies were also performed at an older age.

We agree with the reviewer, and added the following sentence to the abstract:

*"When aged to 12 months, injected R176Q/+ mice maintained a 100% reduction in arrhythmia induction."*

The reviewer suggests revising the following statement: "Unlike other disorders associated with ventricular arrhythmias, implantable cardioverter-defibrillators (ICDs) may increase the risk of sudden cardiac death in CPVT patients due to a higher burden of inappropriate shocks and vulnerability to complications, such as electric storm [3,4]". An ICD might be ineffective in certain subset of arrhythmias in patients with CPVT but the cited original article does not show an increased risk of sudden cardiac death because of ICDs. The second cited reference is a review article and does not contain original data. It would be more accurate – in this reviewer's opinion - to state that ICDs might be ineffective in a subset of patients with CPVT such as those with incessant ventricular arrhythmias.

Thank you for the suggestion. We removed the second reference and changed the wording as suggested.

Figure 1E. If the data is presented as fold change and normalized to Gapdh mRNA, the mean in the control group should be around 1 (instead of close to 1.5). It seems that the data is relative to RQ-con. It should be to wild type.

Thank you for the observation. We have normalized the data to the WT control group and updated Figure 1.

Figure 1F. The differences in the RYR2 protein levels are hard to discern. Would a large panel or a darker exposure show the differences better?

Thank you for the observation. We have changed panel F in figure 1 to another set of mice, that more clearly shows differences.

Regarding the presentation of ventricular arrhythmias, it would be more informative to describe additional characteristics besides the presence or absence of ventricular arrhythmias or the duration. The number of mice in these experiments should be depicted in the figures or stated in the text (whenever not described in the figure legend).

We depicted the number of mice both in the text and the figure. We also described the ventricular arrhythmias as either bidirectional or polymorphic ventricular tachycardia in the text.

The duration of ventricular arrhythmias in the RQ-con seems to be very short (in both sets of experiments), despite pacing electric stimulation and treatment with ISO and caffeine, which casts some shadow on the model. The authors might wish to comment on the shortcomings of the model.

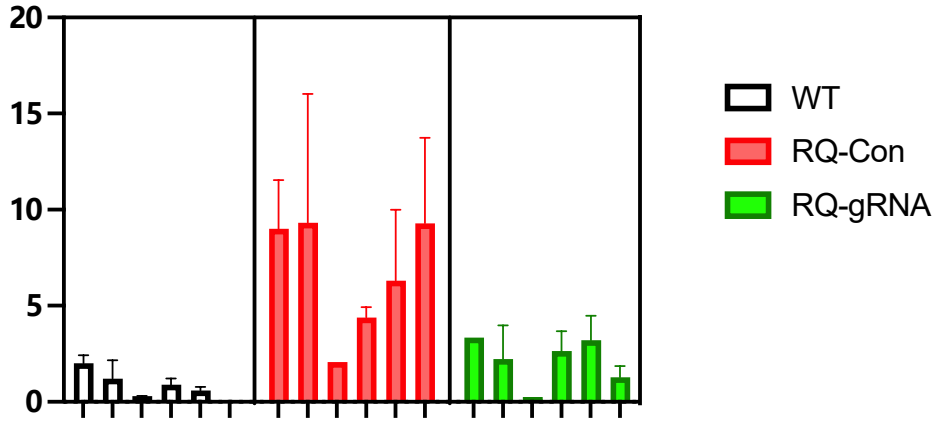
We understand the reviewers concern with the R176Q/+ model. One difficulty with CPVT mouse models is that there are multiple forms of ventricular arrhythmia including bigeminy, polymorphic ventricular tachycardia, and bidirectional ventricular tachycardia. While polymorphic ventricular tachycardia is severe, it is also typically short in duration

<1sec even under isoproterenol. Additionally, the bidirectional ventricular tachycardia seen in CPVT can last for minutes but the way our programmed electrical pacing protocols were run in an automated way, arrhythmias were actually stopped or at least paced over after 1 second. We realize that this did not for an accurate measurement of the duration, and as such, we removed quantifying duration from the figure and replaced it with stating the severity of the ventricular arrhythmia in the text. In the future, we will modify our pacing protocols to better capture the longer durations of BDVT in the R176Q/+ model.

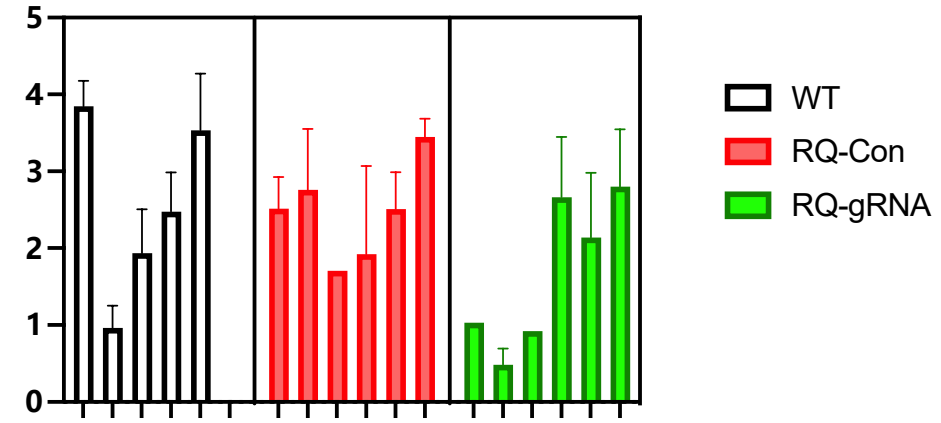
Regarding the data on calcium sparks, there is an issue of independence of the samples in each genotype, meaning that data obtained in several cardiomyocytes from each mouse should be considered as one unit and the mean number per mouse per genotype should be presented and used in data analysis. The reviewer recognizes that this form of presentation is common with these types of studies and there are plenty of studies that have used a similar form of data presentation and analysis. Nevertheless, the readers should be informed that it is incorrect to mix/pool data from multiple cardiomyocytes (20 cells here) obtained from multiple mice (5 or 6 mice) as one group. The approach violates the fundamental principle of independence of the samples mandated by the statistical methods used in these analyses. Likewise, these measurements are extremely soft phenotypes, which are subject to massive underrepresentation (few cells out of several million cells per heart), ascertainment bias, and selection bias and show massive dispersion. The reviewer leaves it to the discretion of the authors to present their data as is but the reviewer encourages the authors to rise above the common trend and either perform the proper statistical analysis that considers the dependence of the samples or present the data per mouse.

We agree with the reviewer in that we should rise above the trend and not perform statistical analysis in pooled data from cardiomyocytes isolated from multiple mice. We have gone back and done a Nested 1-way ANOVA for each of the graphs represented in the figures and changed the P-values. Although, the graphs still represent the pooled cells, the new statistics uses the mouse rather than individual cells. In the manuscript, Figure 3 has been updated. In the supplement, Supplemental Table 2 has also been modified with the new P-values. Below, we have provided the original Nested graphs for the following parameters.

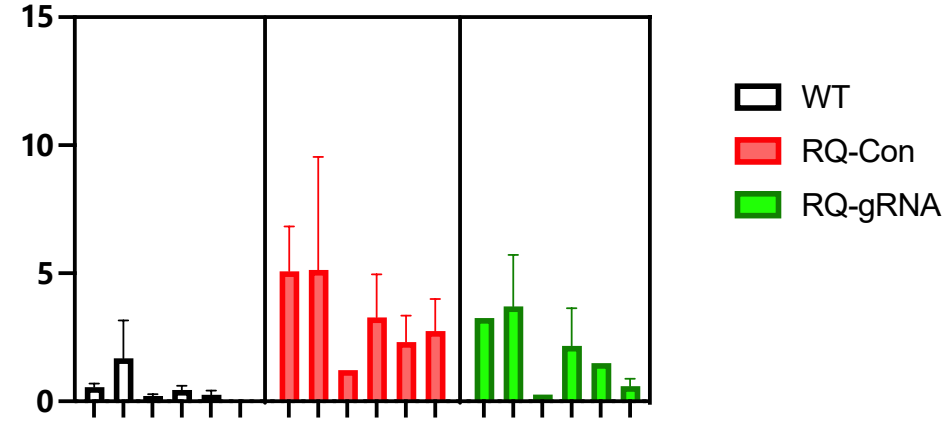
## Nested CaSpF: One-way ANOVA




## Nested SR Load: One-way ANOVA




## Nested CaSpF/SR Load: One-way ANOVA



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Thank you, we have made the suggested edit.

Line 430. Suggest changing solid evidence to evidence (as later it is stated that the study likely underestimated the extent of genome editing).

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**Reviewer 2 Report**

This is a very nice manuscript. The authors have tested gene editing in neonatal (10 days old) mice to suppress the expression of the mutant RyR2. As the disease is heterozygous, the maneuver decreases the RyR2 expression about half, but the expressed channel is the WT protein. This treatment has a protective role against arrhythmias, and the effect is maintained even one year after the injection. I only have minor comments.

Statistics: Although the Methods states that multiple group comparisons are performed, the figures only show pair comparisons. Adding the comparison between RQ treated and WT would be informative. For example, in Fig 3, one wonders if the treated RQ still present more Ca sparks than the WT.

Ca sparks are not really arrhythmogenic. Did you observed/measured Ca2+ waves?

In Figures 2 and 5, the duration of ventricular arrhythmias is provided. As There are arrhythmias only in one group (untreated RQ), I don’ t see the sense of this comparison. It only provides the description of VA arrhythmia duration in the only group that have it. Just passing the duration to the text would be enough in my opinion.

At the end of the discussion some limitations should be added as to the feasibility of gene editing in adults.

**Author Response**

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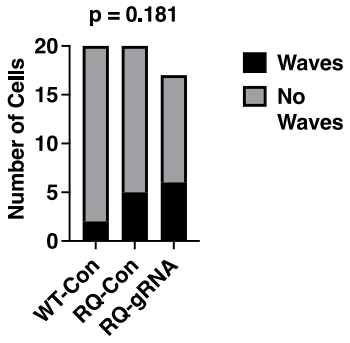
We thank the reviewer for the overall very positive assessment of our manuscript.

Statistics: Although the Methods states that multiple group comparisons are performed, the figures only show pair comparisons. Adding the comparison between RQ treated and WT would be informative. For example, in Fig 3, one wonders if the treated RQ still present more Ca sparks than the WT.

We thank the reviewer for the suggestion. We have added the additional comparison between wild type and genome edited mice in the revised Figure 3. We have also redone the analysis using a nested one-way anova as the samples are not independent.

Ca sparks are not really arrhythmogenic. Did you observed/measured Ca2+ waves?

We quantified the $Ca^{2+}$ waves for the individual groups and found no significant differences in wave incidence between any of the groups according to Chi Square Analysis. See figure below. This study was underpowered to determine the differences in cellular arrhythmogenicity based on waves, so we decided not to include it in the final version.

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We agree with the reviewer and removed ventricular arrhythmia duration from the figures. Due to differences in ventricular arrhythmia, instead of reporting duration we distinguished severity between bidirectional and polymorphic ventricular tachycardia in the text.

At the end of the discussion some limitations should be added as to the feasibility of gene editing in adults.

We have added the following text to page 18.

"An important consideration for future CRISPR/Cas9-based therapies is if the effectiveness of genome editing will translate to older adults. Most preclinical studies in animals have applied treatments at younger ages and then followed these animals to older ages. By example, a recent study on *PCSK9* gene editing for hypercholesteremia has shown durability and effectiveness of liver genome editing starting at 9 weeks of age until 476 days after treatment in non-human primates [PMID 36314243]. The clinical trial for the same *PCSK9* therapy is currently recruiting for ages ranging up to 75 [NCT05398029] and will reveal the relative effectiveness depending on the starting age of the therapy or whether there will be immune reactions to SaCas9 specific to older adults. For the

translation of our approach disrupting mutant RyR2 expression, one concern with older adults is that RyR2 levels may decrease with comorbidities such as diabetes [PMID 11723243] and heart failure [PMID 1829688], in which case an RyR2 replacement therapy might be needed."