Author's response to reviews

Title: Cardiac involvement in Beagle-based canine X-linked muscular dystrophy in Japan (CXMDJ): electrocardiographic, echocardiographic, and morphologic studies

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Author's response to reviews: see over
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To the person who may concern,

The comments of the reviewer has been helpful in allowing us to revise again our manuscript (MS: 1681285926109043) “Cardiac involvement in Beagle-based canine X-linked muscular dystrophy in Japan (CXMDJ): electrocardiographic, echocardiographic, and morphologic studies”, by Naoko Yugeta et al, for publication in BMC Cardiovascular Disorders. We have attempted to address all the questions raised by the reviewer. We have prepared the reply letter to the reviewer and attached. The corrected parts in the manuscript have been indicated by red color.

Thank you for your consideration of the 2nd revised version in advance.

Sincerely yours,

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Reply to the reviewer, Dr. Luca Ferasin:

Major Compulsory Revisions

1. Introduction

The Authors explained in better details their justification for the study in the introduction. However, the sentence “dogs are large” is still present in the abstract and should be changed. Furthermore, the new sentence “In addition, we have gotten beagle bitches and male dogs in Japan, who have clear origins” is confusing and does not add any useful information.

> As the reviewer stated, we have deleted the sentence “dogs are large” and newly added a sentence in the abstract as indicated below. In addition, we have removed the sentence “In addition….clear origins” from the introduction.

The sentence added in the Abstract (p3; line 10):

but GRMD is very difficult to maintain because of their severe phenotypes.

2. Methods

a) Although I appreciate that the Authors changed the title of the paragraph “Electrocardiography”, the method description is still confusing. Presumably, the measured PQ and QRS intervals did not change between leads, so the word “lead III” could be omitted.

> The PR and QRS intervals in leads II and III were almost same. We, therefore, deleted “lead III”, as the reviewer stated.

b) Q/R ratio was calculated in the three limb leads and in one augmented lead (aVF). However, in Discussion, the Authors refer to “peculiar ECG finding in DMD characterized by deep and narrow Q-waves in limb leads I and aVL and left precordial leads V5-6 [13]”. Why was the Q/R ratio analysis made in aVF if the purpose of the study was to identify a suitable animal model which resembles DMD? I would suspect that the Authors have calculated the ratio in all augmented lead but found a significance difference only in aVF. If that was the case, it should be mentioned in the description of the methods. I would suggest the Authors to rewrite the entire paragraph.

> In DMD patients, the distinctive deep Q waves are detected in leads I, aVL, and V5-6
(as indicated in the Introduction) or in leads II, III and aVF [ref. 13]. However in normal control and in CXMD, Q waves were not prominent in leads aVR and aVL. Therefore, we measured the Q/R ratios in leads I, II, III and aVF (Figure 2B) and found the prominent Q/R ratios in leads II, III and aVF. In GRMD, the deep Q waves were also observed in leads II, III and aVF [ref. 23]. The difference of the findings between DMD and dog models might be derived from the relationship between the position of the heart and the location of electrodes. As the reviewer indicated, we have rewritten the paragraph in the Methods.

The electrocardiographic studies (p9):
Leads I, II, III, aVR, aVL and aVF were recorded in the right lateral recumbency using an ECG-922 electrocardiograph (Nihon Koden, Tokyo, Japan) [27]. All ECGs were obtained at a paper speed of 50 mm/sec and calibration of 10 mm/mV. First, the electrocardiography were performed in two CXMD (III-302MA, III-303MA) and one normal littermate (III-301MN) dogs at 2, 3, 4, 6, 9, 12, 15, 18, and 21 months of age, and the heart rate (HR), intervals of PQ and QRS, and Q/R ratios were measured. However, in normal control and in CXMD, Q waves were not prominent in leads aVR and aVL, therefore, we measured the Q/R ratios in leads I, II, III and aVF. Next, we compared the HR, intervals of PQ and QRS, or Q/R ratios in I, II, III and aVF in eight CXMD and four normal dogs at 6-7 months of age.

c). During my first revision, I omitted to request clarifications on why histopathology of the right ventricular myocardium was not performed. A short explanatory sentence would be appropriate.
> We have also examined right ventricular myocardium, but right ventricles were rather intact. We have changed the descriptions in the methods and results as below.

The phrase changed in the Method (p10; line 13):
taken from the left ventricle …> taken from the right and left ventricles…

The sentence changed in the Results (p13; line 15):
The left ventricle walls were …> The right and left ventricular walls were …
The sentence added in the Results (p14; line 4):
We found that the right ventricular walls were kept intact in all CXMD\textsubscript{J} dogs examined.

3. Results

The paragraph “echocardiographic findings” is rather confusing and should be rewritten. I would also recommend the Authors to double check the sequential list of events and lesions in the different subjects.

> As the reviewer pointed out, the paragraph of echocardiographic findings in the Methods and Results were confused. We have, therefore, rewritten the paragraph, corrected Table 2, and newly prepared Table 3. In particular, we summarized echogenicity of left ventricular posterior wall of normal and CXMD\textsubscript{J} dogs under sequential manner in Table 3.

a) The normal value of fractional shortening in dogs is reported as >30%; however this should be referenced. The same comment applies to the legend of figure 2A.

> Crippa L et al. showed that FS in normal male Beagle dog is 40 +/- 10% (mean +/- SD) [Lab Anim 1992;26:190-5]. Therefore, we considered the normal range of FS as more than 30%. We cited the reference (ref. No. 28) and added the comments into the Methods and Figure legend 3A.

b) The Authors state the hyperechoic lesion was first detected in III-302MA at 12 months. However, they mention later that similar lesions were detected in III-D08MA and III-303MA at 5 and 6 months but not in other dogs at 6-7m. This is confusing.

Furthermore, under Methods, the Authors state that echocardiographic examination was performed just before euthanasia, but the chronology does not match with the results and table 2. I would recommend rewrite echocardiographic methods and correct table 2.

> As mentioned above, we have rewritten the Methods and Results, corrected Table 2, and newly prepared Table 3.

The echocardiographic studies in the Methods section (p9)
M-mode and two-dimensional echocardiography was performed using an EUB-8000 echocardiograph (Hitachi Medical Corporation, Tokyo, Japan). The thickness of the interventricular septum (IVS) and left ventricular posterior wall (LVPW) at end-diastole,
left ventricular internal dimension at end-diastole (LVIDd) and systole (LVIDs), and fractional shortening (FS) were examined on normal and CXMDJ dogs using M-mode echocardiography. We considered the normal range of FS as more than 30% based on a previous report [28]. We examined the parameters mentioned above and myocardial echogenicity in two CXMDJ (III-302MA, III-303MA) and one normal littermate (III-301MN) dogs at 2, 3, 4, 6, 9, 12, 15, 18, and 21 months of age. We also examined the parameters mentioned above and myocardial echogenicity in six CXMDJ (III-D53MA, III-D55MA, III-1803MA, III-D38MA, III-D02MA, III-D08MA) and three normal dogs (III-D56MN, III-1804MN, III-D03MN) at the time point just before euthanasia. Among those dogs, myocardial echogenicity in one CXMDJ (III-D02MA) and its normal littermate (III-D03MN), and another CXMDJ (III-D08MA) dogs were also examined at various time points.

The echocardiographic studies in the Results section (p12)
The thickness of LVIDd, IVS, and PW in two CXMDJ (III-302MA, III-303MA) were not different from those in a normal littermate (III-301MN) by sequential analysis with advancing age (Fig. 3A). Those parameters were not different between other six CXMDJ and three normal dogs, when examined just before euthanasia (Table 2). FS in III-302MA decreased with advancing age and was slightly lower than normal value at 21 months of age (Fig. 3A), but FS in the other seven CXMDJ were normal, even just before euthanasia (Table 2). A mild hypokinesis of the left ventricular wall was detected in III-302MA at 21 months of age (Fig. 3B), but any clinical signs had not been developed in the dog.

The sequential studies of myocardial echogenicity with advancing age in III-302MA and in III-303MA demonstrated that the hyperechoic lesions in the left ventricular posterior wall were seen at 12 months of age or older (Fig. 4A, Table 3). In the subsequent examinations of six CXMDJ, we found the hyperechoic lesion in a CXMDJ, III-D08MA, at 5 months of age (Fig. 4B, Table 3), however the hyperechoic lesion was not detected in other four CXMDJ at 5 to 7 months of age (Fig. 4B, Table 3). One CXMDJ, III-D38MA, did not reveal any hyperechoic lesions, when examined at 12 months of age, but has not been examined at 5 to 7 months of age (Table 3). Taken these echocardiographic data, it is considered that the cardiac functions in CXMDJ were
basically maintained well by 21 months of age, despite showing hyperechoic lesions of the left ventricle in limited numbers of CXMD\textsubscript{J}.

c) The fact that clinical signs were not present does not imply that echocardiographic changes were mild. They were mild based on subjective evaluation of a mild regional hypokinesis and near-to-normal FS (although I would still consider 27\% a normal value for FS in dogs).

> As the reviewer mentioned, the lack of clinical signs did not guarantee mild echocardiographic changes. Therefore, we deleted inadequate sentences, as indicated above.

4. Discussion

a) The hyperechoic lesions are mentioned only for the dog III-D08-MA. What about the other two individuals?

> According to the reviewer’s suggestions, we incorporated the data of other two CXMD\textsubscript{J} as indicated below.

**The sentence added in the discussion (p15; line 19):**
The hyperechoic lesion in the left ventricular posterior wall was detected in both III-302MA and III-303MA, but not early as 12 months of age (Table 3).

b) The Authors imply that CXMD\textsubscript{J} dogs may represent a better animal model when compared to GRMD. Although early ECG changes are well documented, other cardiac lesions appear to develop in a milder fashion at a later stage in the CXMD\textsubscript{J} dogs. In my opinion, this may represent a disadvantage, since a longer period of time is needed to obtain significant changes in diseased animal.

> I accepted the reviewer’s comment that mild cardiac phenotypes of CXMD\textsubscript{J} may rather represent disadvantages of CXMD\textsubscript{J} as a disease model. At the same time, however, mild phenotypes may provide a chance to investigate the molecular pathogenesis of the electrocardiographic changes, since we clearly described these changes at the early phase. We, therefore, add the description in the discussion.

**The sentence added in the discussion (p18; line 6):**
---, but we may recognize that a longer period of time would be required to complete cardiac phenotypes in CXMD₁.

c) Other important limitations that the Authors should acknowledge are the low number of animals used in this study, the lack of biochemical monitoring of myocardial damage (i.e. serum troponin-I levels), and the fact that the progression of the disease was not assessed over a longer period of time.

> I basically agree with the reviewer’s comments. Cardiac phenotypes should be observed in more CXMD₁ dogs for a longer period of time with biochemical markers. We have, however, given some considerations to diminish the number of experimental animals based on the advice by the ethics committee. Actually, serum markers for cardiac damage such as troponin-I and -T have been sequentially examined, but the number of CXMD₁ was limited. Therefore, we will report them in the next paper. We have added the sentence in the Discussion as follows.

**The sentence added in the Discussion (p 16; line 19):**

---, although a longer period evaluation of large numbers of CXMD₁ will be needed to conclude the mild cardiac phenotypes of CXMD₁.

**Minor Essential Revisions**

1. Figure 3A: Please report a reference for >30% as normal FS value in dogs

> We have cited a reference, as shown above (3a).

2. Figure 4: Figure 3B and figure 4 might be merged. Alternatively, the Authors should consider reporting only the pictures of dogs with the observed echocardiographic lesions. Please explain in the text the meaning of the arrowhead (i.e. hyperechoic lesions).

> As the reviewer recommended, we have merged Figure 3B into Figure 4 and renamed old Figure 3C as new Figure 3B. We explained in the Figure legend that the arrowhead indicated hyper-echoic lesion.

3. Figure 5: Figure 5 is very elegant. However, the Authors should also report the sample origin for III-D55MA and III-D02MA (or at least explain it in the legend)
We have added an explanation of the origin for III-D55MA and III-D02MA in the legend.

**The sentence added in legends for Figure 5:**
Posterior walls of left ventricles of both III-D55MA and III-D02MA were macroscopically normal (data not shown).

4. Figure 6: Figure 6 implies that 100% of CXMDj dogs would present hyperechoic lesions and fibrotic changes at 20 months of age. This does not match with the results presented in this study. Please verify and correct. Furthermore, the significance of the dot-line is not explained.

We have regarded the percentage of hyperechoic lesion and fibrotic change in CXMDj at 18 months of age or older as 100%, because both CXMDj (III-302MA and III-303MA, showed in Table 3) at 18 months of age or older had the lesion. I, however, noticed that the number of examined CXMDj was small (n=2), at 18 months of age or older, therefore we gave dot-line for the portion of percentage. The meaning of dot-line has been explained in the legend for Figure 6.

**Discretionary Revisions**

There are some grammatical imperfections that need to be corrected before the editorial review. Line numbering would have helped. A short list of suggested changes is reported below:

1. Page 3, Methods, 2nd line: “were” instead of “was”
2. Page 4, Conclusion, 1st line: “…are milder and have slower progression than those described in GRMS dogs”.
3. Page 5, 2nd paragraph, line 6: “…In DMD patients, the electrocardiogram (ECG) may show tall R waves…”
4. Page 6, line 8: “…the reason why the posterobasilar…”
5. Page 6, last line: the first letter of common breed names (such as Beagles) should be written in capital font.
6. Page 8, line 5: “…dogs were recently described [25]”. (remove “in our recent paper”)

> We have changed these sentences as the reviewer corrected.
7. Page 8, line 13: “unnecessary” instead of excessive  
> We have changed “excessive” into “unnecessary”.

8. Page 9, under ECG and Echocardiography: “just before euthanasia” instead of “just before autopsy”  
> We have changed the phrase “just before autopsy” into “just before euthanasia”.

9. Page 9, ECG, last line: “calibration”, instead of “voltage standardization”  
> We have changed the phrase “voltage standardization” into “calibration”.

10. Page 9, Echocardiography, line 5: standard abbreviation for left posterior ventricular wall is LVPW (instead of PW)  
> We have changed the abbreviation “PW” to “LVPW”.

11. Page 9, Echocardiography, line 7: fractional shortening is abbreviated as %FS. Later in the manuscript is abbreviated as FS. Please choose one or the other.  
> We have selected FS.

12. Page 10, 3rd line: “…underwent cardiac histological…”  
> We have added “cardiac”.

13. Page 10, last line: “none of the dogs in the present study showed…”  
> We have changed the sentence as the reviewer suggested.

14. Page 11, 1st line: please change “symptoms” into “clinical signs”.  
> We have changed “symptoms” into “clinical signs”.

15. Page 11, line 5: Please remove “on the other hand”  
> We have removed the conjunctive.

16. Page 11, line 5: “Serum CK levels in the CXMDj dogs ranged from 12,500 to 138,000 IU/l. These values were significantly different…”  
> We have changed the sentence as the reviewer addressed.
17. Page 11, line 8: Please remove “(Table 1)”
> We have deleted “Table 1”.

18. Page 11, ECG findings, 1st line: “intervals” instead of “interval”
> We have changed “interval” to “intervals”.

19. Page 11, ECG findings, 1st line: “different from those recorded from”
> We have changed the sentence as the reviewer indicated.

20. Page 11, ECG findings, 2nd line: “littermates” rather than “littermate”
> We left the word “littermate” based on the comment of the reviewer #1 on the original version of manuscript.

21. Page 11, ECG findings, 3rd line: “15 months of age”
> We have changed “15 months” into “15 months of age”.

22. Page 11, ECG findings, 4th line: “intervals” instead of “interval”
> We have changed the word “interval” into “intervals”.

23. Page 11, ECG findings, 5th line: “were” rather than “was”
> We left the word “was” based on the comment of the reviewer #1 on the original version of manuscript.

24. Page 11, ECG findings, 5th line: “correlated both in normal and affected dogs”
> We have changed the sentence as the reviewer suggested.

25. Page 11, ECG findings, 7th and 9th line: “littermates” instead of “littermate”
> We left the word “littermate” based on the comment of the reviewer #1 on the original version of manuscript.

26. Page 13, Discussion, 1st line: Please remove (PR)
> We have removed “(PR)”.