Sequence variation in PPP1R13L results in a novel form of cardio-cutaneous syndrome


Corresponding authors: Tzipora Falik-Zaccai and Orly Avni, Galilee Medical Center

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Transaction Report:

(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. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Roberto Buccione

1st Editorial Decision 01 June 2016

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three Reviewers whom we asked to evaluate your manuscript.

We are sorry that it has taken longer than usual to get back to you on your manuscript. In this case, we experienced significant difficulties in securing three expert and willing Reviewers. Further to this the evaluations were delivered with some delay and, finally, the decision required further discussion.

As you will see, although Reviewers 1 and 3 are more appreciative of your study, all three raise significant and fundamental issues that in aggregate, I am afraid, preclude publication of the manuscript in EMBO Molecular Medicine at this time. I will not discuss each point in detail as they are clearly stated.

The main issues, notwithstanding different perceptions on the overall interest of the study, include 1) the lack of clear casual evidence linking the in vitro observations to the in vivo phenotype, and 2) the need for further experimentation (including on the mouse models) to provide sufficient mechanistic insight, especially in terms of the how the pro-inflammatory phenotype is causing fatal DCM.

Given these fundamental concerns and the general lack of enthusiasm by the Reviewers, I have no
choice but to return the manuscript to you at this stage. I'm afraid that we agree with their opinions and in our assessment it is not realistic to expect to be able to address these issues experimentally and to the satisfaction of the Reviewers in a reasonable time frame.

I wish to add however that, considered the potential interest of these findings, we would have no objection to consider a new manuscript on the same topic if at some time in the near future you have obtained data that would considerably strengthen the message of the study and address the Reviewers' concerns.

I am sorry to have to disappoint you at this stage and again apologise for the inevitable delay. I hope that the Reviewers' comments will be helpful in your continued work in this area.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

The ppp1r13l knockout mice are known to exhibit wavy hair and progressive dilated cardiomyopathy (DCM) phenotypes. Falik-Zaccai et al found genetic variation in PPP1R13L can cause a novel form of cardio-cutaneous syndrome in human. This is interesting findings, and the genetic evidence is strong.

However, I found the mechanistic links are weak: how pro-inflammatory leads to dys-regulation of DCM related genes? The authors should provide mechanistic insights how PPP1R13L affects NF-κB signaling and subsequent expression of DCM related genes.

Referee #2 (Remarks):

This manuscript is a case report of several children who developed DCM and also harbor a loss of function of the PPP1R13L gene. The data primarily make the case for an association of the genetic variation and the phenotype, and causality cannot be established based on the studies presented. The strongest evidence is the phenotype of KO mice which have a KO of the PPP1R13L gene, which resembles features found in the affected cases. Knockdown studies in cardiac fibroblasts and neonatal cardiomyocytes suggest that effect on inflammatory signaling pathways might underlie the onset of DCM. However, there is no direct evidence that the onset of the DCM phenotype in vivo relates to these in vitro findings and studies in the KO mouse model would strengthen this claim, both at the molecular pathway level, and in terms of blocking the onset of DCM.

Without these data, the study is primarily a case study with a few additional suggestive results that might infer pathways that link genotype and phenotype. Taken together, my view is that it should be published somewhere but probably better for a specialty journal like European Heart Journal.

Referee #3 (Remarks):

In recent years an increasing number of genetic risk factors for cardiomyopathies have been determined, thereby elucidating the pathogenesis of previously idiopathic cardiomyopathies. In this report the authors present strong and novel evidence that a severe childhood cardiomyopathy in consanguineous patients is caused by defective gene PPP1R13L. In addition to an inflammatory dilated cardiomyopathy (DCM), the patients present with distinct skin and eye phenotypes, thereby classifying this disorder as cardio-cutaneous syndrome (CCS). The same combination of symptoms has been previously described in PPP1R13L deficient mice. PPP1R13L is known to regulate/suppress aspects of NFκB-mediated inflammatory signaling. The present work presents novel evidence that a PPP1R13L defect (or shRNA knockdown) indeed leads to an enhanced inflammatory cytokine expression in the CCS context, i.e. in patient-derived fibroblasts or mouse cardiomyocytes/hearts. The enhanced expression of inflammatory mediators is especially enhanced upon challenge of cells by inflammatory stimuli, i.e. LPS. These data are highly suggestive, however, formal proof is lacking that this hyper-inflammation is indeed causing the deadly DCM in the patients. This is my major concern about this work, which could be addressed experimentally in
the PPP1R13L deficient mouse. As described by Herron et al (2005) these mice develop DCM, however, the mice are able to live at least 8 months. Would a low dose LPS challenge of younger mice (i.e. 6 wk old) trigger the development of DCM as compared to PPP1R13L deficient mice kept in the fairly clean environment of an animal facility? Such an experiment would not only elucidate the pathogenesis of DCM but would also have clinical impact on management of such patients by suggesting an anti-inflammatory/anti-bacterial strategy. Please discuss such strategies.

Besides this major comment I have a few minor points:
1) Figure legends do not match the panels presented, i.e. Figure 1.
2) Figure 1 is very complex and should be broken into more figures and more clearly presented.
3) Figure 5 A presents mouse / heart pictures that have been reported more convincingly and in more detail before (Herron 2005). I suggest omitting those pictures and focus on inflammation and DCM as in my major comment.
4) page 6/line 2: What is "AR CCS"?

1st Revision - authors' response 10 October 2016

Thank you for the insightful review of our manuscript, and for your offer to reconsider a new version of our manuscript on the same topic.

In this new version of the manuscript we present data that strengthens the main point of the manuscript, i.e.: that absence of PPP1R13L results in hyper-sensitivity to inflammatory triggers, and as a consequence, leads to fatal dilated cardiomyopathy (DCM).

Our study was appreciated by the reviewers, but they had some concerns that were summarized by you as follows:
1. the lack of clear casual evidence linking the in vitro observations to the in vivo phenotype
2. the need for further experimentation (including on the mouse models) to provide sufficient mechanistic insight, especially in terms of how the pro-inflammatory phenotype is causing fatal DCM.

We would like to address these concerns point by point.

1. "The lack of clear casual evidence linking the in vitro observations to the in vivo phenotype"
In the previous version we showed that patients' derived fibroblast and ppp1r13l-knocked down cardiomyocytes demonstrated significant hyper-sensitivity to inflammatory stimulus. In this new version of the manuscript we clearly link this phenomenon to the cardiac phenotype of the murine model, carrying spontaneous mutation in ppp1r13l (wa3 mice). The new data, which are presented in a new section in the Results entitled: "Dynamic development of inflammatory transcriptional patterns in ppp1r13l-deficient hearts" (including new Figures 6-7, Figures 3S-7S, and Tables 1S-3S), demonstrate in vivo dynamic alterations in cardiac transcriptional programs in the absence of ppp1r13l during DCM development. In this section, by using RNA-seq for newborn, 7 week- and 12-week-old wa3-derived hearts, we clearly show the development of inflammatory conditions, starting with differential expression of only one gene; the s100a9 in newborns. S100a9 is known to be associated with heart inflammation (Averill, Kerkhoff et al., 2012). From that point, we observed a gradual (less in 7-week old mice and much more in 12-week old mice) wa3-selective increase in the expression of pro-inflammatory mediators, such as those associated with cytokines-cytokine receptors, chemokine-chemokine receptors, TOLL-like receptors (TLRs), complement, phagocytosis, cell adhesion, T cell function and proliferation, TGFb signaling, and NF-kB signaling pathways. To further study in vivo the cardiac response to inflammatory triggers in the absence of Ppp1r13l, LPS was injected to 12-week-old wa3 mice, and the results of the RNA-seq analysis indicated a higher inducible expression than in controls of genes associated with acute inflammation. In accordance, two consecutive weekly injections of low amount of LPS demonstrated a lower resistance of wa3 mice in response to the inflammatory stimulus; almost all of the mutants died during these two weeks while none of the controls. Although our results cannot exclude predisposition of the wa3 hearts to inflammatory triggers, all together our data indicate a crucial requirement for ppp1r13l in lifting the cardiac threshold response to inflammatory triggers. In its absence, low doses of inflammatory stimuli are fatal.
"The need for further experimentation (including on the mouse models) to provide sufficient mechanistic insight, especially in terms of the how the pro-inflammatory phenotype is causing fatal DCM"

The involvement of inflammatory cytokines in DCM promotion is quite established for many years, although the exact molecular mechanisms underlying this causative effect are not completely understood. We mentioned it in the second paragraph of the Discussion: "The idea that over-expression of typical pro-inflammatory mediators such as the cytokines IL-1β, TNFa, and IL-6, as we found in the absence of iASPP, leads to DCM is in accordance with established data showing that the presence of these mediators in plasma, as well as in the myocardium itself, potentiate cardiac remodeling processes such as hypertrophy, ventricular dilation, fibrosis and apoptosis (Dick & Epelman, 2016, Gullestad, Ueland et al., 2012, Prabhu & Frangogiannis, 2016)". Actually, expression of pro-inflammatory mediators and the inducible activity of NF-κB downstream are initially cardio-protective, since early inflammatory functions are necessary for the transition to later proper physiological reparative program, but prolonged activation can lead to sustained tissue damage and improper healing, defective scar formation, heightened cell lose and contractile dysfunction, which eventually promote DCM. Therefore, our results demonstrating overall increased expression of genes associated with inflammation, fibrosis and tissue repair, are most probably the reason for the early appearance of the fatal DCM (before age 6 month). Our results can promote the development of better diagnostic tools for dissecting the stage of the disease and most importantly new therapeutic approaches, to prevent and/or to alleviate the development of the disease.

Referee#1
Referee#1 agreed that we demonstrated interesting findings, and that the genetic evidence is strong. However, Referee#1 found that the mechanistic links are weak: "how pro-inflammatory leads to dysregulation of DCM related genes?" As we explained above the connection between inflammation and consequent fibrosis and deleterious reparative machinery and DCM is well established (Dick & Epelman, 2016, Gullestad et al., 2012, Prabhu & Frangogiannis, 2016), although the exact molecular mechanisms and the sequence of dynamic alterations have not totally been dissected yet, and can be case-specific. Our results contribute insights into the sequential events toward DCM, and may suggest future stage-specific markers for diagnosis, prevention, and case-specific medical intervention.

Referee#2
Referee#2 claimed "that Knockdown studies in cardiac fibroblasts and neonatal cardiomyocytes suggest that effect on inflammatory signaling pathways might underlie the onset of DCM. However, there is no direct evidence that the onset of the DCM phenotype in vivo relates to these in vitro findings and studies in the KO mouse model would strengthen this claim, both at the molecular pathway level, and in terms of blocking the onset of DCM".

As we mentioned above, our new data demonstrated the development of inflammatory conditions in vivo in the absence and even stronger in response to inflammatory triggers, and that external inflammatory trigger is lethal for wa3 mice.
Referee#3
We were pleased to realize that Referee#3 thinks that "In this report the authors present strong and novel evidence that a severe childhood cardiomyopathy in consanguineous patients is caused by defective gene PPP1R13L…The present work presents novel evidence that a PPP1R13L defects (or shRNA knockdown) indeed leads to an enhanced inflammatory cytokine expression in the CCS context, i.e. in patient-derived fibroblasts or mouse cardiomyocytes/ hearts. The enhanced expression of inflammatory mediators is especially enhanced upon challenge of cells by inflammatory stimuli, i.e. LPS."

However, the major concern of Referee#3 about our work was that "formal proof is lacking that this hyper-inflammation is indeed causing the deadly DCM in the patients…which could be addressed experimentally in the PPP1R13L deficient mouse. As described by Herron et al (2005) these mice develop DCM, however, the mice are able to live at least 8 months. Would a low dose of LPS challenge of younger mice (i.e. 7-week old) trigger the development of DCM as compared to PPP1R13L deficient mice kept in the fairly clean environment of an animal facility? Such an experiment would not only elucidate the pathogenesis of DCM, but would also have clinical impact on management of such patients by suggesting an anti-inflammatory/anti-bacterial strategy. Please discuss such strategies."

As mentioned above, we fully agree with Referee#3 and therefore performed, with success, the suggested experiment. As can be seen in Figure 7, LPS injections increased the expression of pro-inflammatory mediators more strongly in the hearts of wa3 mice than controls, and two sequential weekly injections of LPS were lethal selectively for wa3 mice.

Regarding discussion of therapy options, we added the following paragraph to the Discussion: "Although experimental work has established a crucial role for the inflammatory cascade in cardiac repair and remodeling, to date, there has been no successful clinical immunomodulatory or anti-inflammatory therapeutic strategies for heart diseases (Prabhu & Frangogiannis, 2016). Since the reparative response is a highly dynamic process, as can be learned from the alteration in the transcriptional patterns during diseases development, a successful immunomodulatory therapy should require careful design implying knowledge on the time course of the inflammatory process and the temporary associated immune cells. The pathophysiologic heterogeneity based on the genetic background of patients with heart diseases has also important therapeutic implications. e.g. patients with impaired iASPP function may be predispose to dilative remodeling after myocarditis or myocardial infarction, and therefore benefit from personalized tailored anti-inflammatory intervention."

Response to the minor points:
1) Figure legends do not match the panels presented, i.e. Figure 1. Sorry, the Figure legend has been corrected
2) Figure 1 is very complex and should be broken into more figures and more clearly presented. Figure 1 was split into Figure 1 and Figure 2
3) Figure 5A presents mouse/heart pictures that have been reported more convincingly and in more detail before (Herron 2005). I suggest omitting those pictures and focus on inflammatory and DCM as in my major comment. We decided to leave this Figure since the murine background between these two manuscripts is different C57bl/6 (Herron 2005) and Balb/C (this manuscript). We moved the Figure to Supplementary Data Figure 2S, and mentioned that these results were presented previously by Herron 2005.
4) page 6/line 2: What is "AR CCS"?
Autosomal Recessive (AR) CCS, mentioned firstly in Results page 6.

Again, we fully understand that we are submitting our manuscript as a NEW SUBMISSION that includes new data that were requested by the reviewers and yourself. We hope that our work will now be determined suitable for publication in the journal EMBO Molecular Medicine.
References:


Dick SA, Epelman S (2016) Chronic Heart Failure and Inflammation: What Do We Really Know? Circulation research 119: 159-76


Prabhu SD, Frangogiannis NG (2016) The Biological Basis for Cardiac Repair After Myocardial Infarction: From Inflammation to Fibrosis. Circulation research 119: 91-112


Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final editorial amendments.

You may refer to the author guidelines (http://embomolmed.embopress.org/authorguide) for details on how to address most of the following. Do not hesitate to contact the editorial office if you need further help.

***** Reviewer's comments *****

Referee #2 (Remarks):

I think the authors have addressed my initial concerns...I am still a bit underwhelmed by their mechanistic data and the links to the in vivo murine model context, but their experimentation does strengthen their case.

Referee #3 (Comments on Novelty/Model System):

This second version of paper is of high quality throughout. The "medium" novelty is only due to the fact that a DCM in the mouse model had been described 10 years ago. Here comes the relevance for people ... and this should fit to EMBO Mol Med.

Referee #3 (Remarks):

well done - no further comments
Authors made the requested editorial changes.
EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: trải Faïk-Zacca

Journal Submitted to: EMBO Molecular Medicine

Manuscript Number: EMR-2016-06521

Reporting Checklist for Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- The data were obtained and processed according to the field’s best practices and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- Figures panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- Graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- If n=1, the individual data points from each experiment should be plotted and any statistical test employed should be justified.
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- A specification of the experimental system investigated (e.g. cell line, species name).
- The assay(s) and method(s) used to carry out the measured observations and measurements.
- An explicit mention of the biological and chemical entity(ies) that are being measured.
- An explicit mention of the biological and chemical entity(ies) that are altered/perturbed in a controlled manner.
- The exact sample size (n) for each experimental group/condition, given as a number, not a range.
- A statement of how many times the experiment was independently replicated in the laboratory.

Definitions of statistical methods and measures:

- Are tests one-sided or two-sided?
- Are there adjustments for multiple comparisons?
- Which statistical test results, e.g., P values, were used?
- Are there any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g., randomization process)?
- Was the sample size chosen to ensure adequate power to detect a pre-specified effect size?
- If yes, please describe.
- If not why?
- Were any steps taken to minimize the effects of subjective bias when analyzing animal samples to treatment (e.g., randomization procedure)?
- If yes, please describe.
- If not why?

B- Statistics and general methods

1. a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?
   
   The sample size was chosen to achieve an acceptable p-value. The biological repeats were usually performed in triplicate.

1. b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.
   
   For animal tests, we used the minimum number of mice that was sufficient to achieve statistical significance in RNA-Seq with the acceptable p-value threshold.

2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?
   
   Yes, the analysis was conducted from the analysis. Page 16.

3. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g., randomization process)? If yes please describe.
   
   Yes, we used a blinded investigator to assign the groups.

4. For animal studies, include a statement about the randomization even if no randomization was used.
   
   Yes, we used a blinded investigator to assign the groups.

5. How were the data analyzed and presented? If you used any additional methods please describe.
   
   We used a t-test for all the experiments with the Tukey’s multiple comparisons.

6. Did the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess this.
   
   Yes, we used a t-test for all the experiments with the Tukey’s multiple comparisons.

7. Were there outliers removed within each group of data?
   
   Yes, we used a t-test for all the experiments with the Tukey’s multiple comparisons.

8. Was there a variance in the groups that were statistically compared?
   
   Yes, we used a t-test for all the experiments with the Tukey’s multiple comparisons.

9. Are the definitions of center values used median or average?
   
   Yes, we used a t-test for all the experiments with the Tukey’s multiple comparisons.

C- Reagents

- Are the definitions of reagents used standard definitions? (Page 16, figure legend section)

D- Additional information

- Are the definitions of survival or measurements of cytokines in RT-PCR.

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D. Animal Models

- For experiments involving animals, include a statement of compliance with standards/ regulatory agencies and identify the committee(s) approving the experiments.
- Data deposition in a public repository is mandatory for:
  - Proteins, DNA and RNA sequences
  - Macromolecular structures
  - Crystallographic data for small molecules
  - Functional genomic data
  - Promiscuous and molecular interactions.
- For human studies, provide a statement confirming that informed consent was obtained from all subjects.
- We recommend consulting the ARRIVE guidelines (see link list at top right) (Franklin, 2009) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under ‘Reporting Guidelines’. Please confirm compliance.
- For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have followed these guidelines.
- 中文.
- E. Human Subjects

- Identify the committee(s) approving the study protocol.
- Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.
- For publication of patient photos, include a statement confirming that consent to publish was obtained.
- Report any restrictions on the availability (and/or on the use) of human data or samples.
- Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.
- For phase I and II uncontrolled clinical trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have followed these guidelines.

F. Data Accessibility

- Provide accession codes for deposited data. See author guidelines, under ‘Data Deposition’.
- Data deposition in a public repository is mandatory for:
  - Proteins, DNA and RNA sequences
  - Macromolecular structures
  - Crystallographic data for small molecules
  - Functional genomic data
  - Promiscuous and molecular interactions.
- Data deposition is strongly recommended for any datasets that are central and integral to the study, please consider the journal’s data policy. If you structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under ‘Expanded View’ or in a structured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).
- Access to human clinical and genetic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access controlled repositories such as NCBI (see link list at top right) or figshare (see link list at top right).
- Include a statement confirming that data are included.

G. Dual use research of concern

- Include a statement confirming that data are included.
- Ensure that all data are included and that the final manuscript does not differ from the deposited data.
- To ensure that data are included, please provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile e.g., Antibodiesdirect (see link list at top right).
- Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

* For all hyperlinks, please see the table at the top right of the document.

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