Author's response to reviews

Title: Increased expression of NF-AT3 and NF-AT4 in the atria correlates with procollagen I carboxyl terminal peptide and TGF-beta1 levels in serum of patients with atrial fibrillation

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Author's response to reviews: see over
Dear Editors and Reviewers:

Thank you for your letter and the Reviewers’ comments concerning our manuscript “Increased expression of NF-AT3 and NF-AT4 in the atria correlates with procollagen I carboxyl terminal peptide and TGF-β1 levels in serum of patients with atrial fibrillation” (MS: 1099015766137793). All the incisive comments were very helpful for improving our paper as well as clearly stating the significance of our research. The revised text is in red font. Our point-by-point responses to the Reviewers’ comments are as follows:

**Editorial comments/request:**

We recommend that you copyedit the paper to improve the style of written English.

**Response:** We have had the manuscript edited by a professional editing company using native English speakers.

Also, please label your Competing Interests and Authors’ Contributions section accordingly.

**Response:** We have added Competing Interests and Authors’ Contributions sections. (Page 22)

**Reviewer #1:**

The authors should carefully organize their figures. Most figures and figure legend are mismatched. The label used in the figure should be consistent with it in the legend, such as Fig.1 and Fig.2

**Response:** We have reorganized our figures to match the figure legends and made the labels in figures consistent with the legends.

Please spell out abbreviation at first use in abstract, article, and figure legends

**Response:** We have added a table of abbreviations, and have spelled out abbreviations at first use in the manuscript.

Please indicate the extent of magnification in Fig.1 and Fig.2

**Response:** We have added the extent of magnification in the Methods section and in the figure legends.
Please reorganize Fig.3, Fig.4, Fig.5. Please indicate which panel is the representative blot, which one is the quantification of the blot.

Response: We have reorganized these figures and indicated the representative blot and the quantification of blot.

What's the mechanism that nuclear NF-AT3 and NF-AT4 participates in atrial structural remodeling? Is NF-AT3 and NF-AT4 involved in the atrial structural remodeling by increasing TGF-#1 level, then inducing PICP secretion and collagen synthesis? Please clarify it.

Response:

We have added the following explanation to the revised manuscript: “The transcription factors NF-AT3 and NF-AT4 are the downstream effectors of calcineurin, and play an important role in the calcineurin-dependent pathway during cardiac hypertrophy. Recent studies also directly and indirectly implicate the calcineurin-dependent pathway in the development of cardiac fibrosis.” (Page 6, lines 17–20) In our study, we found clinical evidence for a relationship between nuclear NF-AT3 and NF-AT4 and atrial fibrosis in atrial fibrillation. We also wanted to determine sensitive serum biomarkers in the blood to establish the extent of atrial remodeling in atrial fibrillation. Future studies are required in our studies to determine the definite mechanism by which nuclear NF-AT3 and NF-AT4 participate in atrial structural remodeling.

Reviewer #2:

The authors talked a lot about the NF-AT3/4, but there not background introduction of these two important genes. They mentioned a little bit about their roles in the calcineurin signaling pathway. Actually both are important transcription factors that regulate cytokine genes in T cells (nuclear factor of activated T cells), as well as cardiac genes. I would suggest the authors give a little bit more background about these genes, and what we already know their roles in cardiac disease.

Response: Thank you for your comment. We have added more details/research articles on these two important genes into the Background section of the revised manuscript.
P13: a little background of PeAF, PaAF and SR would help readers understand all association assays. (e.g. clinical symptom: PeAF>PaAF>SR). In addition, it would be nice to show atrial images of each category so that the differences in atrial sizes can be appreciated.

Response: We have added the following explanation of PeAF, PaAF and SR into the revised Methods to describe their associations: “The patients were divided into three groups: sinus rhythm (SR; n = 30), persistent AF (PeAF; AF lasting >6 month, n = 30), and paroxysmal AF (PaAF; recurrent AF that terminated spontaneously in <7 days, n = 30).” (Page 8, lines 5–8). We have insufficient evidence to demonstrate significant differences of clinical symptoms among these three groups and there are many factors affecting clinical symptoms. We have hemodynamic and echocardiographic data of every patient. Showing atrial images of each category is a good way to show the differences in atrial sizes, which we plan to do in the future.

Table 1 has a lot of value information that can be talked in more details in the result (e.g. disease condition, drug taken, NYHA class etc.) or in the method. It would be worthwhile to study the correlation between these conditions to AF gene expression too.

Response: Table 1 shows all clinical information about different groups and we want to remove possible factors affecting the results to find the relationship between NF-AT3/4 expression and atrial fibrosis. We found that the constituent ratios of patients with different heart function were not significantly different among the three VHD groups. We have added this explanation to the revised manuscript (page 14, lines 9–11).

Meanwhile, we found that left atrial diameter, measured by echocardiography, was significantly larger in the PeAF group than in the SR group. Furthermore, the left atrial diameter was significantly larger than the right atrial diameter in all groups, except the control group (page 14, lines 4–7). We also identified the different valvular disease as a factor affecting the results and did analysis in the section of Result. We plan to analyze correlations between drug taken and AF gene expression in the future.

P14: Line 10-12 and line 13-16 are repetitive. They just said the same result twice. Actually there
is so much information in the figures 3-5, but they were not presented properly in the text. Maybe the author need describe in more detail on the comparison. Additionally, how was the nuclear NF-AT3/4 expression assay performed? I didn't read anything about the assay in the method.

Response: Thank you for your comment. We have deleted lines 13–16 as we found that NF-AT3/4 expression increasing in the atria with atrial fibrillation has been previously reported, and these findings were not key to our study. The objective of this study was to determine the relationship between NF-AT3/4 expression and atrial fibrosis and serum fibrosis biomarkers. In the revised Methods section, we have added a description of the expression assays and tissue collections (page 10, lines 22; page 11, lines 1–9).

Regarding to the correlation analysis, I feel figures 6-8 are not very necessary. To be honest, the information I only looked was the R and p value. I would suggest that tables are good enough. Also, what is the summary for each correlation analysis? They seem all correlate with each other, then what’s the point?

Response: We have deleted figures 6–8 and summarized correlation analysis in a table. Increased expression of NF-AT3/4 in the atria with atrial fibrillation has been previously demonstrated by other studies. However, the relationship between NF-AT3/4 and atrial fibrosis have not been verified. We performed analysis on correlations between NF-AT3/4 mRNA and nuclear protein and collagenI/III mRNA and protein in the atria to determine whether expression of NF-AT3/4 correlates with atrial fibrosis in patients with atrial fibrillation(Table 3).

P17: My same concern is about the last NF-AT3/4 expression essay in different valvular disease. The author simply listed all comparisons. Then what’s the point? What messages do they want to deliver?

Response: The objective of this experiment was to analyze NF-AT3/4 expression in different valvular disease, to determine whether different valvular disease will affect the expression of NF-AT3/4. In the Discussion, we have provided further analysis of the impact of increased NF-AT3 and NF-AT4 expression in the PeAF group compared with that in SR group with similar valvular disease. We also found that NF-AT3 and NF-AT4 expression in patients with pure mitral valvular disease was increased compared with that in patients with pure aortic valvular disease in PeAF, PaAF, and SR groups. Therefore, we have added a discussion point that atrial fibrillation and valvular disease (particularly
MVD) can affect NF-AT3/4 expression. Thus, atrial fibrillation may be an important factor affecting NF-AT3/4 expression and atrial remodeling.

P18-19: The first two paragraphs in discussion are just background. They can be put in the introduction. The discussion should be talking about the discovery and the interpretations, the potential mechanisms, and the clinic applications.
Response: We have reorganized the Background section and moved the content of the first two paragraphs of the Discussion to the Background. We have added additional interpretations of our data in the revised Discussion.

Table 3 and figures 3-4 are repetitive. Table 4 and figure 5 are repetitive. Figures should be good enough.
Response: We have deleted Tables 3 and 4.

Table 6 and 8 doesn’t have any data.
Response: We have added the data to Tables 6 and 8; these have been renumbered as Tables 4 and 5.

It seems there some format issues on all figures. There are 15 pages of figure, but figures are only numbered to 8. Some pages should be combined.
Response: We have reorganized the figures.

In the end, personally, I would argue the semi-qRT-PCR is not very reliable for mRNA quantification. A Real-time PCR would be the best way for future mRNA quantification studies.
Response: We have also recently found that real-time PCR is better for mRNA quantification, and plan to use it in future studies.

P1: title: “Increased expression of NF-AT4 and NF-AT3 in the atria correlates…” should be “Increased expression of NF-AT3 and NF-AT4 in the atria correlates…”
Response: We have changed the title.

P2: Line 9: “Atrial fibrillation is the most common cardiac arrhythmia…” should be “Atrial fibrillation (AF) is the most common cardiac arrhythmia…” It the first time to define AF.
Response: We have changed this sentence.

TGF-#1. TGF-beta1, TGF-# should all be unified to the same format.
Response: We have unified these to the same format (TGF-β1).

P6: line 13: “We excluded four categories of patients from this study”. I only see three categories.
Response: We apologize for our oversight. We have added the text “(iv) patients with fibrosis disease that could affect serum fibrosis biomarkers.” (Page 8, lines 14–15)

P7: line 21: 4% paraformaldehyde?
Response: Yes, it is 4% paraformaldehyde. This has been corrected in the revised manuscript.

P9: line 14: which lysis buffer did you use?
Response: We use RIPA as lysis buffer. This has been added into the revised manuscript. (Page 11, line 1).

P10: line 10: “the” should be “then”
Response: We have changed “the” to “then”. This sentence has been changed in the revised manuscript, and now reads, “The membranes were then incubated for 2 h at 37°C with secondary antibodies.” (Page 11, lines 21–22).

P13: line 10: “Right atrial diameters were not significantly different among the three VHD groups.” It is repetitive with the prior sentence
Response: We have deleted this sentence.

P14: line 16: FigureS3B should be Figure3B.
Response: We have deleted “S”.

P21: line 14: “Further” should be “Furthermore”.
Response: We have changed “further” to “Furthermore”.

For all tables, I’m confused by the small superscript characters. What do they really mean?
Response: The small superscript characters are to show paired comparison among different groups. For example, in table2, we mark superscript “a” in TGF-β1 and PICP average value because there are significant difference between PeAF+VHD group and PaAF+VHD group and we can see that the average value of TGF-β1 and PICP in PeAF+VHD group are obviously more than PaAF+VHD group. We do not mark superscript “a” in PIINP and PINP average value because there are not significant differences between PeAF+VHD group and PaAF+VHD group. Although the average value of TGF-β1 and PICP in PeAF+VHD group are more than PaAF+VHD group, there are not statistical significance.

In figure1 and 2, a sale bar is need
Response: We have added a scale bar in figures.

We wish to thank you and the Reviewers for your time and effort. We hope that the revised manuscript is now suitable for publication.