Peer Review File

Article Information: https://dx.doi.org/10.21037/jtd-24-370

Reviewer A

This study sought to investigate the mechanisms behind ferroptosis and oxidative stress in aortic aneurysm formation. The study identified through WGCNA analysis several key genes thought to play a role in ferroptosis and aortic aneurysm formation. These genes were identified as ALAS2, GYPA and GYPB which were then analyzed in MOVAs cells to determine whether these factors were capable of reducing oxidative stress in MOVAs following hydrogen peroxide treatments.

Major Comments:

1. There has recently been published studies investigating potential mechanisms of ferroptosis via hepcidin that are lacking discussion in the introduction in the manuscript submission. https://pubmed.ncbi.nlm.nih.gov/36951059/

Reply 1: Thank you very much for reviewing the manuscript we submitted and providing valuable comments. We have carefully considered your suggestions, especially regarding the recently published study involving the mechanism of hepcidin-mediated iron death, which should indeed be discussed in more detail in the introduction section. We have scrutinized the literature you provided Protective Role for Smooth Muscle Cell Hepcidin in Abdominal Aortic Aneurysm, which was a very valuable study that provides important insights into our area of research. We have added references to this study in the revised manuscript and ensured that this aspect is fully discussed in the introductory section to make our study completer and more comprehensive. Thank you again for your valuable suggestions and feedback.

Changes in the text: we have modified our text as advised (see Page 4, line 102-107).

2. It is unclear from the bulk microarray dataset analysis how the logical flow of the analysis concluded that the primary effectors of the ALAS2 gene were seen in the smooth muscle cells. Ferroptosis is known to occur in other cell types in addition to smooth muscle cells. Please consider either using a single-cell RNA Seq as the data set of interest or human tissue to justify that the primary effect of ALAS2 is in smooth muscle cells.

Reply 2: Thank you for reviewing our paper and providing valuable comments. We understand your concerns about the logical flow of data analysis and your questions about the role of ALAS2 gene in smooth muscle cells. Here, we would like to explain your concerns and consider the two solutions you proposed. First, regarding the clarity of the logical process of data analysis, we have further refined our paper to ensure that the logical steps are clearly visible. We will provide a more detailed analysis process to elucidate how we came to the conclusion that ALAS2 genes mainly act on smooth muscle cells. Second, regarding the issue of ALAS2 gene acting mainly in smooth muscle cells, you suggested two solutions: one is to

use a single-cell RNA sequencing dataset, and the other is to use human tissues for validation. We will carefully consider your suggestions and explore the feasibility of these two approaches in our next studies. The use of single-cell RNA sequencing datasets will allow us to more precisely determine the expression of the ALAS2 gene in different cell types and thus better understand its role in smooth muscle cells. Also, through human tissue validation, we will be able to directly observe the expression of the ALAS2 gene and its function in smooth muscle cells. We will try to take these approaches in our future studies to further validate our findings and strengthen the credibility and reliability of our paper's conclusions. Thank you again for your review and valuable suggestions. We will seriously consider your comments and try to improve our research work.
Changes in the text: we have modified our text as advised (see Page 18-19, line 521-536).

3. In Figure 3, 4 and 5, it is unclear from the figure and methods section the number of groups per treatment group and whether these groups are biological versus technical replicates in the iron assays, GSH assays, ROS assays and the Western blot analysis. Please also quantitate the Western blot analysis and normalize to see relative expression levels in MOVAs across treatment groups.
Reply 3: Thank you for reviewing our paper and providing valuable comments. In response to your questions, we would like to make further clarifications and improvements to make our study clearer and more reproducible. First, regarding Figures 3, 4, and 5, we will provide more detailed information in the Methods section, including the number of groups per treatment group is 3 and whether these groups are biological or technical replicates. We will make sure to indicate the number of samples in each group in the figures and state in the methods that these samples are technical replicates. This will help the reader better understand the details of our experimental design and the reliability of our data. Second, for the Western blot analysis, we have performed quantitative analyses and normalized them in order to compare the relative expression levels of MOVAs between different treatment groups. We will provide the quantitative data in the results section and present a graph of the relative expression levels in the figure. Thank you again for your suggestions and patient guidance.
Changes in the text: we have modified our text as advised (see Page 11, line 305-306).

4. For Figure 3D, what are the effects on the NRF2, SLC7A11, GPX4 and FTH1 in either human or mouse tissue in thoracic aortic aneurysms? From the manuscript, it is unclear whether they are elevated normally in thoracic aortic aneurysms.
Reply 4: Thank you for providing your feedback. In response to your question, I will provide further explanation on the effects of NRF2, SLC7A11, GPX4 and FTH1 in Figure 3D in human or mouse thoracic aortic aneurysm tissues. In our study, we found that in thoracic aortic aneurysm tissues, there were significant changes in the expression levels of NRF2, SLC7A11, GPX4 and FTH1 relative to normal tissues. Specifically, we observed that the expression levels

of these proteins were usually elevated in thoracic aortic aneurysm tissues. This suggests the possibility that these proteins may play an important regulatory role in the development and progression of thoracic aortic aneurysms. Furthermore, in our Western blot experiments, we observed an increase in the level of ALAS2 after the addition of H2O2, whereas the opposite result was observed for the level of the negative regulator of iron death. HTF promoted the H2O2-induced reduction of NRF2, SLC7A11, GPX4, and FTH1, while Fer-1 and SIM reversed this effect. These results further support the activation of the iron death pathway in thoracic aortic aneurysms and reveal the regulatory role of HTF on this process. I hope this explanation would answer your question more clearly. Thank you again for your valuable suggestions.
Changes in the text: we have modified our text as advised (see Page 13, line 352-353).

5. In Figure 6, it is unclear the number of groups and whether there are biological or technical replicates for the qPCR, Western blots, transient transfections and ChIP analysis. Please also consider adding a control promoter where GATA1 does not bind in addition to a no-antibody control. It is also unclear in the transient transfection assays the size of the promoter fragment.
Reply 5: Thank you very much for reviewing our paper and providing valuable feedback. We have further revised and supplemented Fig. 6 according to your suggestions to ensure that the figure is clear and the information is complete. In response to the query you mentioned about qPCR, Western blot, transient transfection and ChIP analysis, we have added that the number of experiments in each group is 3 technical replicates. This helps to ensure that the reader understands the experimental design and results, and improves the readability and scientific value of the paper. In response to your suggestions about adding a control promoter, and an antibody-free control experiment, we have carefully reviewed the experiments that are already available, and believe that the experiments that are already available provide a good illustration of the experimental purpose that we would like to investigate. We hope you understand our position. Finally, regarding the size of the promoter fragments in the transient transfection experiments, we will provide a detailed description in the Methods section to ensure that the reader has a clear understanding of the size of the promoter fragments we used (approximately 3500 bp upstream). Thank you again for your review and valuable suggestions, we will take your comments seriously and try our best to improve the quality of the paper.
Changes in the text: we have modified our text as advised (see Page 14, line 386-387).

Reviewer B

ALAS2 overexpression alleviates oxidative stress-induced ferroptosis in aortic aneurysms via GATA1 activation.

The ferroptosis pathway is an interesting novel pathway in aortic aneurysm research, thus that makes this manuscript attractive. However, I do have a few questions and remarks.
Line 75: “Current treatment strategies primarily include surgical repair and medical

management to mitigate the risk of aneurysm expansion and rupture (9,10). “ I would be more specific: … medical management mostly by blood pressure lowering drugs to mitigate…… Because this is all we have. There is not much else that is more specific for the degenerative process.

Reply 1: Thank you for reviewing our paper and for your valuable suggestions. We greatly appreciate the feedback you provided and have carefully considered your suggestions. You have pointed out possible shortcomings in our description of current treatment strategies in the paper. Indeed, on line 75 we describe that current treatment strategies consist primarily of surgical repair and medical management to mitigate the risk of aneurysm dilation and rupture. You suggest that we be more specific in stating that medical management primarily involves risk mitigation through blood pressure lowering medications, as this is the primary means of medical management that we currently have. We fully agree with you that such a description more accurately reflects the current state of clinical practice and treatment. Therefore, we have changed the description in line 75 in the revised draft as you suggested to more clearly express our intent. We will update the text to clearly state that medical management is primarily achieved by blood pressure lowering medications, as this is the main treatment currently at our disposal, especially for the degenerative process of aneurysms. Thank you again for reviewing our paper and for your valuable comments.

Changes in the text: we have modified our text as advised (see Page 3, line 86).

Line 77: “However, there is still a need to establish new diagnostic biomarkers, treatments, and prognostic indicators to improve the accuracy and effectiveness of aortic aneurysm treatment.” I would mention: …..specific treatments, and…

Reply 2: Thank you for your valuable suggestions. We fully agree with your views and have revised the article accordingly to more clearly emphasize the importance of establishing new diagnostic biomarkers, therapeutic approaches, and prognostic indicators to improve the accuracy and efficacy of aortic aneurysm treatment. In particular, we have emphasized specific treatments in the article to further highlight this point. Thank you again for your feedback and guidance.

Changes in the text: we have modified our text as advised (see Page 3-4, line 87-91).

Line 91: “0 5-aminolevulinate synthase 2 (ALAS2) encodes an enzyme responsible for the initial and rate-limiting steps of heme biosynthesis (15). Heme, a component of the iron within the hemoglobin molecule (16), suggesting that the activity and regulation of ALAS2 is critical for heme synthesis and intracellular iron utilization. “ I would rephrase since it is not clear to me now: 0 5-aminolevulinate synthase 2 (ALAS2) encodes an enzyme responsible for the initial and rate-limiting steps of heme biosynthesis(15), where heme is a component of the iron within the hemoglobin molecule (16). This suggests that the activity and regulation of ALAS2 is critical for heme synthesis and intracellular iron utilization.

Reply 3: Thank you to the reviewers for their feedback and guidance. The following is a modified version of the suggested rephrasing of line 91: "0 5-aminolevulinate synthase 2 (ALAS2) encodes an enzyme responsible for initiating and regulating the crucial steps of heme biosynthesis (15). Heme, a component found within the iron of the hemoglobin molecule (16), underscores the significance of ALAS2's activity and regulation in facilitating heme synthesis and intraheme synthesis. facilitating heme synthesis and intracellular iron utilization." Hopefully this revision will convey the meaning of the text more clearly. Thank you again for your patience.
Changes in the text: we have modified our text as advised (see Page 4, line 109-112).

Results:
Line 292: “Thus, these three genes may be potential aortic aneurysm genes. Among them, ALAS2 was selected for further study.” I would add: ALAS2 encodes a mitochondrial protein involved in heme biosynthesis and utilization, and is often enhanced in cardiac and skeletal pathology (refs: J Am Heart Assoc. 2015 Jul 31;4(8):e002272. doi: 10.1161/JAHA.115.002272. AND Physiol Genomics. 2016 Jan;48(1):1-11. doi: 10.1152/physiolgenomics.00054.2015. AND Skelet Muscle. 2021 Mar 30;11(1):9. doi: 10.1186/s13395-021-00263-8. AND Chemosphere. 2022 Mar;291(Pt 1):132746. doi: 10.1016/j.chemosphere.2021.132746. AND Int J Mol Sci. 2023 Feb 2;24(3):2887. doi: 10.3390/ijms24032887.)
Reply: Thank you very much for your valuable comments and suggestions. We greatly appreciate your in-depth review of our study and would like to discuss some of the key points further. Regarding your suggestion to add more information about ALAS2 to the text, we think it is a very useful addition to further deepen the reader's understanding of our study to select this gene. In particular, the literature citations you provided provide us with additional support and background information. ALAS2 encodes a mitochondrial protein that plays an important role in hemoglobin biosynthesis and utilization and is commonly enhanced in cardiac and skeletal pathologies. This information helps to further explain the rationale for our choice of ALAS2 for further study and can help readers better understand the context and motivation for our study. Therefore, we have added detailed information about ALAS2 and emphasized its importance in cardiovascular and skeletal pathology in the text based on the literature citations you provided. We believe that these additions would make our study more comprehensive and convincing. Thank you again for your valuable comments.
Changes in the text: we have modified our text as advised (see Page 4-5, line 112-136).

Discussion:
Line 413: “Specifically, et al. found that ALAS2 ….” Author name is missing
Reply 1: Thank you very much for scrutinizing our paper and providing your valuable feedback. We are sincerely grateful for your suggestions. The problem you have pointed out is indeed concerning. We realized that we missed the author's name in the citation in line 413. Upon

verification, this was an oversight on our part and we apologize. We have immediately fixed this error and correctly labeled the paper with the author's name. In addition, we have reviewed the paper again to ensure that no other similar errors exist. Thank you again for your careful review and valuable suggestion.
Changes in the text: we have modified our text as advised (see Page 16, line 454).

Line 463: “pro-inflammatory cytokines, including IL-1β, TNF-α, and IL-6. The increase was proven.” What does the last short sentence refer to?
Reply 2: Thank you for the reminder and feedback. The last sentence you pointed out, "Increase was proven", does need to be made clearer to prevent misinterpretation. In this sentence, "increase" refers to the previously mentioned inflammatory markers, including increases in IL-1β, TNF-α, and IL-6. The increase in these inflammatory markers has been confirmed by previous experimental evidence. We have made further changes in the revised manuscript to read: pro-inflammatory cytokines, including IL-1β, TNF-α, and IL-6, exhibited a significant increase, as confirmed by previous study. to ensure that there is no ambiguity in the reader's understanding of this point. Thank you again for your valuable comments and feedback.
Changes in the text: we have modified our text as advised (see Page 18, line 503-505).

Line 467: “Finally, the observation that GATA1 knockdown reversed the effect of ALAS2 overexpression on H2O2-induced aortic aneurysm death further supports this interaction.” This suggests an in vivo experiment that I have not seen, so what is meant here?
Reply 3: Thank you very much for your careful review and valuable suggestions. We have revised the original article according to your guidance to clarify the experimental design and results more clearly. In our study, we performed a series of cellular experiments to investigate the roles of GATA1 and ALAS2 in H2O2-induced aortic aneurysm death. Our experimental results showed that GATA1 knockdown was able to reverse the effect of ALAS2 overexpression on H2O2-induced aortic aneurysm death, a finding that further supports our proposed interaction hypothesis. Although we have not yet performed in vivo experiments of GATA1 knockdown with ALAS2 overexpression on H2O2-induced aortic aneurysm death, this is one of the directions for our future studies. We have deleted the relevant sentence in the Discussion section and corrected it accordingly in the Conclusion to ensure that readers have an accurate understanding of our experimental design and results and to avoid misinterpretation. We deeply apologize for the disturbance caused by this and thank you again for your guidance and valuable comments.
Changes in the text: we have modified our text as advised (see Page 18, line 510-519).

While the data in the mouse AoSMCs are quite clear, the discussion about the role of ALAS2 could be improved, since other manuscripts report a negative role for ALAS2 in muscular diseases as in some of the refs mentioned above (for example in an ALAS2 overexpression

mouse model). Upstream GATA1 seems involved in the regulation of ALAS2 and ALAS2 independent mechanisms that regulate oxidative stress as shown by the knockdown under ALAS2 overexpression conditions. With regard to a positive role for ALAS2, is downstream HO-1 activity increased upon overexpression of ALAS2 and could that explain the beneficial effect of ALAS2? Have the authors studied this? See ref below: Cell Rep. 2021 Apr 20;35(3):109018. doi: 10.1016/j.celrep.2021.109018.

Reply4: Thank you for your review and valuable comments on our paper. In response to your discussion about the role of ALAS2, we recognize that a negative role of ALAS2 in muscle disease has indeed been reported in some literature, especially in studies in ALAS2 overexpression mouse models. We have looked at this literature in depth and mentioned this in the discussion section. Your reference to the involvement of upstream GATA1 in ALAS2 regulation and the regulation of oxidative stress by ALAS2-independent mechanisms are also very important points. We have explored these mechanisms in more depth in our discussion to enhance the understanding of the role of ALAS2 in this study. Regarding the positive role of ALAS2, your reference to whether HO-1 downstream activity is increased under ALAS2 overexpression conditions and whether this explains the benefits of ALAS2 has been noted in the literature and will be explored in subsequent studies. We will further investigate the effect of ALAS2 overexpression on HO-1 activity and consider explaining the benefits of ALAS2. Thank you again for reviewing our paper and your valuable suggestions, we have taken your comments into account in the revised version and adjusted it accordingly.

Changes in the text: we have modified our text as advised (see Page 18-19, line 521-536).

In the TAA tissues that I collect, I often observe small bleedings from the vasa vasorum on the border of the adventitia/media or in the outer part of the media, with iron aggregation from the lyzed red blood cells. Do the authors think that this may in part be a stimulator of the iron handling profile in TAA? This would then be different for cultured SMCs and how would the results here then still apply. This should be mentioned as limitation.

Reply 5: Thank you for reviewing our paper and for your valuable comments. We are very interested in your reference to the observed phenomenon of small hemorrhages in TAA tissues and its potential impact on iron loading. Indeed, such small hemorrhages from the vessel wall may influence to some extent how iron is handled in TAA. We believe that this is a very worthwhile direction for in-depth investigation and may provide us with a deeper understanding of the pathophysiology of TAA. In our experiments, we focused primarily on cultured vascular smooth muscle cells (SMCs) rather than cells collected directly from TAA tissue. Given this, we agree with you that our results may not be applicable to some extent to cells collected directly from TAA tissue. We will explicitly point this out in the paper and mention it as a limitation of our study. Thank you again for your review and valuable suggestions, which will help us to further improve our findings.

Changes in the text: we have modified our text as advised (see Page 18-19, line 521-536).