

Particulate Matter and Co-occurring Genetic Risk Induce Oxidative Stress and Cardiac and Brain Alzheimer's Pathology

Corresponding Author: Professor Federica del Monte

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Communications Biology.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Butler et al examined the effects of 3 months of PM2.5 vs air control experiments on cardiac and brain proteinopathy and oxidative stress in male and female APP^{swe}/PSEN1^{DE9} double transgenic and C57Bl/6J WT control. The authors suggested that environmental pollution as a potential key contributor to the progression of heart and brain proteinopathy, delineating a crucial time point for early interventions to limit multiorgan damage in vulnerable patients.

The significance of the area of investigation is high, and the idea of understanding how early exposure at 3 months of age in these mice impacts AD progression and heart health would provide insights into biomarkers etc and if sex differences existed. However, there are concerns with sample size and the lack of power, from which the authors have, in many areas, embellished their data interpretation thereby affecting the conclusions. Indeed, many of these data are driven by outliers, with several trend statements from which conclusions are made.

Examples of this reflect the following:

"Similar to the ELISA data, western blot analysis of male heart samples showed a trending increase in Ab42 level in AD mice relative to WT ($t(1, 13) = -1.99, p=0.068$) (Fig. 1D; S2A-B)." However, this reported trending increase is driven by a single mouse outlier, and other data in figure 1F have high variability driving the interpretation due to the small sample size explored. Further, the inability to report female data is a significant limitation of the study.

Similarly, this comment is driven by 1 or 2 outliers with the vast majority of samples in the WT range. "Instead, there was a trending increase in Ab42 specific oligomer signal (using VIA oligomer structural antibodies) in AD mouse hearts ($t(1, 29) = -1.92, p=0.064$) (Fig. 2B, S3)". It is also difficult to interpret the significant increase in total plaque in male brain samples when 60% of the samples have normal values (similar to WT males) and the other 40% are elevated. See figure 3A

Once again it is also difficult to interpret the significance here when most of the sample is similar to FA and the increase is driven by 2 mice. "Although there was no effect of exposure on whole plaque growth (Fig. 3E-G), plaques that were smaller in size before exposure (less than 250 μ m²) grew more than plaques that were originally larger in size (greater than 1000 μ m²) ($t(1, 300) = -1.64, p<0.001$) (Fig. 3H)."

There are also concerns with the cognitive data. The group are making statements based on trends, which are driven by outliers as it comes to the cognitive changes in Fig 5. "PM2.5 exposure, rather than genotype, influenced the results of the Novel Object Recognition Test (Fig. 5G). PM2.5 exposed mice had a trending association of decreased total exploration of objects during the habituation phase of the assay ($t(1, 69) = -1.71, p=0.092$) (Fig. 5H)"

Over statements are once again driven by outliers "Interestingly, a detectable level of oxidized glutathione (GSSG), although not significant, was observed only in the AD-PM2.5 mice but not in the other groups (Fig. 6F)." As such the conclusion that "This data suggests that in the heart, PM2.5 exposure leads to deleterious changes in redox homeostasis and antioxidant

responses.” reflect more of an over-embellishment of data interpretation.

As such conclusions should be tempered down.

Although the authors have run the required sex by group interactions and found in several cases where no sex differences were found, thus they grouped the male and female data. However, given the variability in data, and the sex-reported data in other data, I would want to see the figures broken up by sex. Further, the figure legends need to show how many male and female samples are explored, currently, the author only reports the total sample size.

Given that the echo data were collected under light anesthesia it is important to report the heart rate for the mice as that may be the driver for the slight differences noted in CO between groups.

As the group is exploring the effects of PM2.5 at younger ages, it is important that in the discussion the authors reflect this, given that the AD model typically are indistinguishable from nontransgenic mice on cognitive tasks at six months, the authors should point out how their data reflects others at a young age etc. And if others have reported sex differences in the AD cognitive changes at that age.

Reviewer #2

(Remarks to the Author)

The relationship between cardiac failure and Alzheimer's disease (AD) is an interesting and active research area. A β deposition has been found in the heart of AD patients and patients with heart failure, which raised the term cardiac amyloidosis. AD often co-occurs with heart failure and the two diseases share some common risk factors. However, the association of poor heart health condition with AD and their interaction is rather complicated and can be modified by environmental factors independent of aging. This paper demonstrated that exposure to particulate matter (PM) may have an impact on both the heart and the brain amyloidosis.

The manuscript has a clear emphasis on the effects of PM2.5 exposure on AD pathology in both transgenic AD mice and WT mice. The study was well designed with proper controls and clear data presentation. What is more interesting is the male vs female sex differences comparison in both AD markers and cognitive functional evaluation. Overall, the conclusions were supported by the data provided in the manuscript. However, a few things can be improved to make the paper more convincing.

1. Although the author acknowledged the differences in the chemical composition of PM for male and female mice exposure, it was not clear how this huge variation was introduced. Were they exposed sequentially during different months of the year? Also, the timeline in Supplemental Fig. 1A is a little confusing. Looks like the exposure was just around 60 days or so in this graph but the authors stated in the paper that the PM exposure was 3 months long. Some clarification/correction seems necessary.

2. The manuscript mentioned that a few AD-related phenotypes (such as hyperactivity and anxiety) were not changed post PM2.5 exposure. The authors believe that this is due to the young age of the mice (6 months old) evaluated in this study. It will be more convincing to provide some references using the same 2x Tg AD mouse model to back up this claim. Again, it might also be due to the relatively short PM2.5 exposure if the mice were only exposed for 2 months.

3. The analysis of sex differences was very interesting in this study, although the authors did not observe any sex differences in behavior and functional measurements. Sexual dimorphism in AD patients is very obvious, but these differences in animal models can be model specific with the 3xTg AD model being the most prominent one (Age and gender differences for the behavioral phenotypes of 3xTg Alzheimer's disease mice, Brain Research, Volume 1762, 2021; Dennison, Jessica L. et al. 'Sexual Dimorphism in the 3xTg-AD Mouse Model and Its Impact on Pre-Clinical Research'. 1 Jan. 2021 : 41 – 52.). Some expanded discussion on this topic might be interesting to certain readers.

4. The running title might be more appropriate by replacing Cardiac Alzheimer's by Cardiac Amyloidosis

Reviewer #3

(Remarks to the Author)

The manuscripts focused on the associate of PM2.5 and heart failure/AD, which is important for those researchers in such field. It is interesting and meaningful. The manuscripts might be considered to be accepted after some revision.

1. The components of PM2.5 play an important role on their toxicity, the author should give some discussion on the components.

2. Considering the PM2.5 was more easily enter into lung, the author proved that the PM2.5 was associated with the AD and heart failure, is there any evidence that PM2.5 could enter into brain or heart?

3. Would the biochemical indicators of the blood be affected by the PM2.5?

4. Did the structure of the brain affected by the PM2.5? the author may provide some brain tissue slices.

5. Why the author only evaluates three months effects on mice, not more longer time?

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have done well to address my concerns.

Reviewer #2

(Remarks to the Author)

This work by Butler et al concerns the complex interactions of genetics x environmental factors in the condition of a cardiac Alzheimer's disease, and the topic of research is important and interesting. While mainly focusing on the early stage or a relative shorter period of exposure to airborne particulate matter, the authors did demonstrate air pollutants as one of potential risk factors to the development or progression of heart and brain proteinopathy. The revised manuscript has greatly improved data accuracy as well as the clarity in communication. I'm pleased that the authors have addressed all my concerns and suggestions.

Reviewer #3

(Remarks to the Author)

The present study focuses on the effects of particulate matter towards heart and brain, which is meaningful and interesting for the full understanding of PM on human health. In comparison with previous versions, the revised manuscript has been improved significantly. The data in the manuscript could support its conclusion. Moreover, the manuscript has been carefully revised according to the reviewers' suggestion. However, there is still some issues that should be addressed.

In the manuscript, some discussion on the entrance of PM into brain should be added.

In my opinion, the manuscript could be accepted after the revision.

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Reviewers'

comments:

We would like to thank the reviewers for their comments, and we appreciate that our manuscript was acknowledged for the high significance of the area of investigation.

We are thankful for the opportunity to submit this revised manuscript. We have made every effort to address the feedback and critiques, thanks to which we believe the manuscript is stronger. The revisions are indicated in blue, and all critiques are addressed herein.

Reviewer #1 (Remarks to the Author):

Butler et al examined the effects of 3 months of PM2.5 vs air control experiments on cardiac and brain proteinopathy and oxidative stress in male and female APPswe/PSEN1DE9 double transgenic and C57Bl/6J WT control. The authors suggested that environmental pollution as a potential key contributor to the progression of heart and brain proteinopathy, delineating a crucial time point for early interventions to limit multiorgan damage in vulnerable patients.

The significance of the area of investigation is high, and the idea of understanding how early exposure at 3 months of age in these mice impacts AD progression and heart health would provide insights into biomarkers etc and if sex differences existed.

We appreciate Reviewer 1 highlighting the impact of our study in particular on the importance of investigating the early exposure effect and sex differences.

- 1) However, there are concerns with sample size and the lack of power, from which the authors have, in many areas, embellished their data interpretation thereby affecting the conclusions. Indeed, many of these data are driven by outliers, with several trend statements from which conclusions are made.

We appreciate Rev.1's comments regarding possible outliers influencing the results. A statistical expert was consulted before the submission and the original statistical analyses included the evaluation of all statistical assumptions including the consideration of outliers and no serious violations were noted. While there were some instances where an observation might be considered a possible outlier, these were retained as there was no strong evidence that the values were erroneous nor were they sufficiently unusual to warrant removal. However, given the concern of the reviewer, we now have conducted sensitivity analyses excluding these cases and found that the removal of these observations did not change the results presented in the manuscript. While power may seem limited, we adopted the sample size based on the power resulting from the available literature. The limited exposure period of 3 months might underly the variability we see, with individual differences in early disease progression. Longer exposure periods and older mice is likely to reduce the variability across data sets. We have included a paragraph in the limitations discussing the variability in the findings, the possible outliers and the reviewer's remark of the importance of the early findings for biomarkers (Page 25, lines 9-20). As Rev.1's suggested, we have tempered down the language regarding the conclusions made with trending results across all figures throughout the manuscript. Although not included in the

manuscript, we include below the figures without the outliers. Blue asterisk indicates the panels without outliers. As shown in the figures, the removal of outlier confirmed, and in some instances strengthen the results.

Figure 1

Original Figure

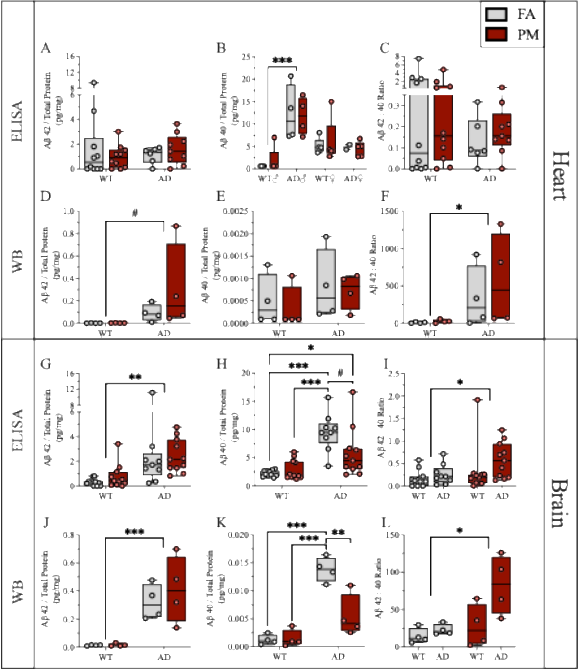


Figure without outliers

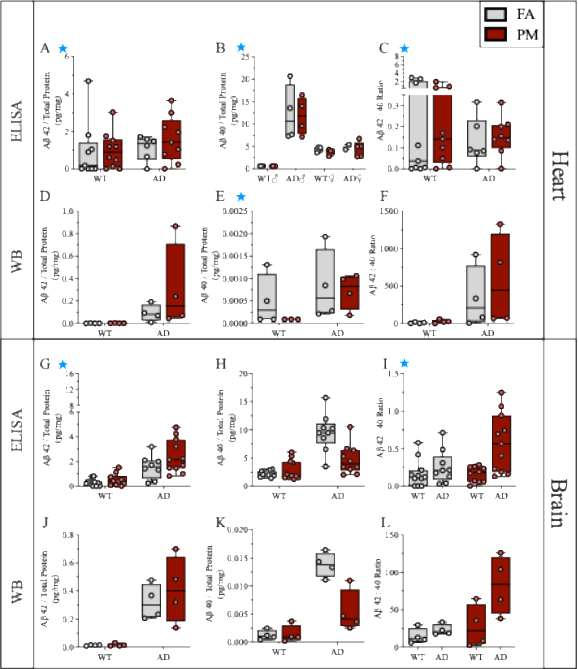


Figure 2

Original Figure

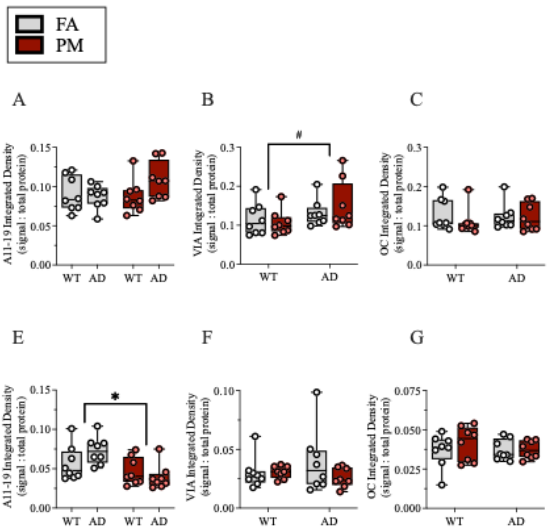


Figure without outliers

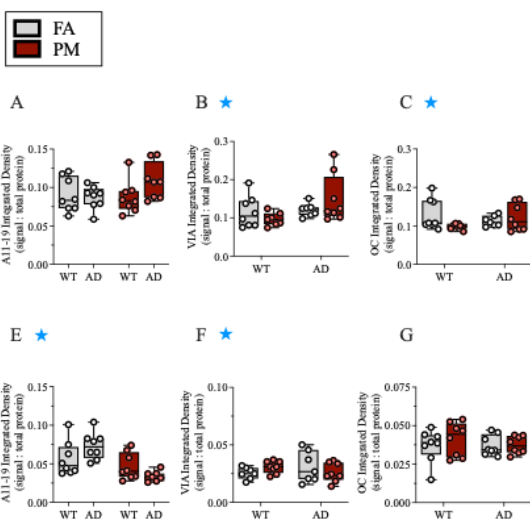


Figure 3

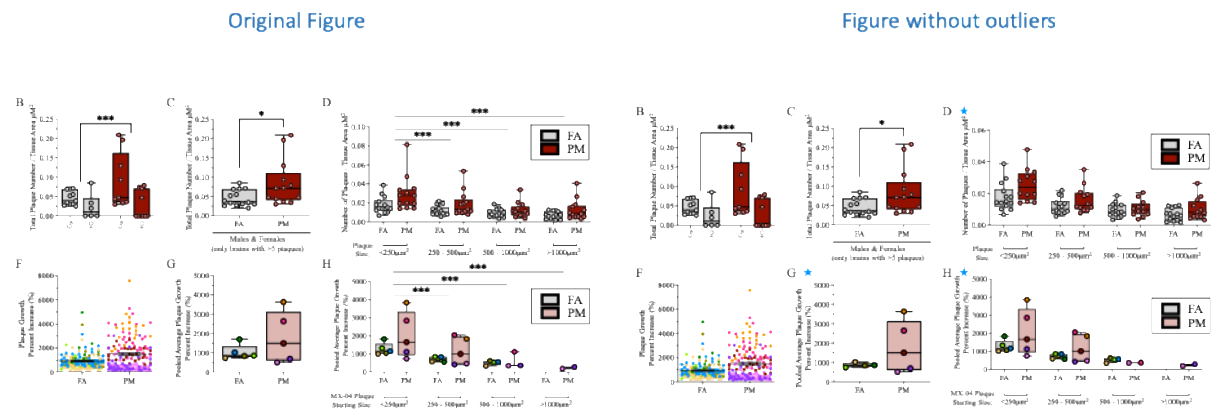


Figure 4

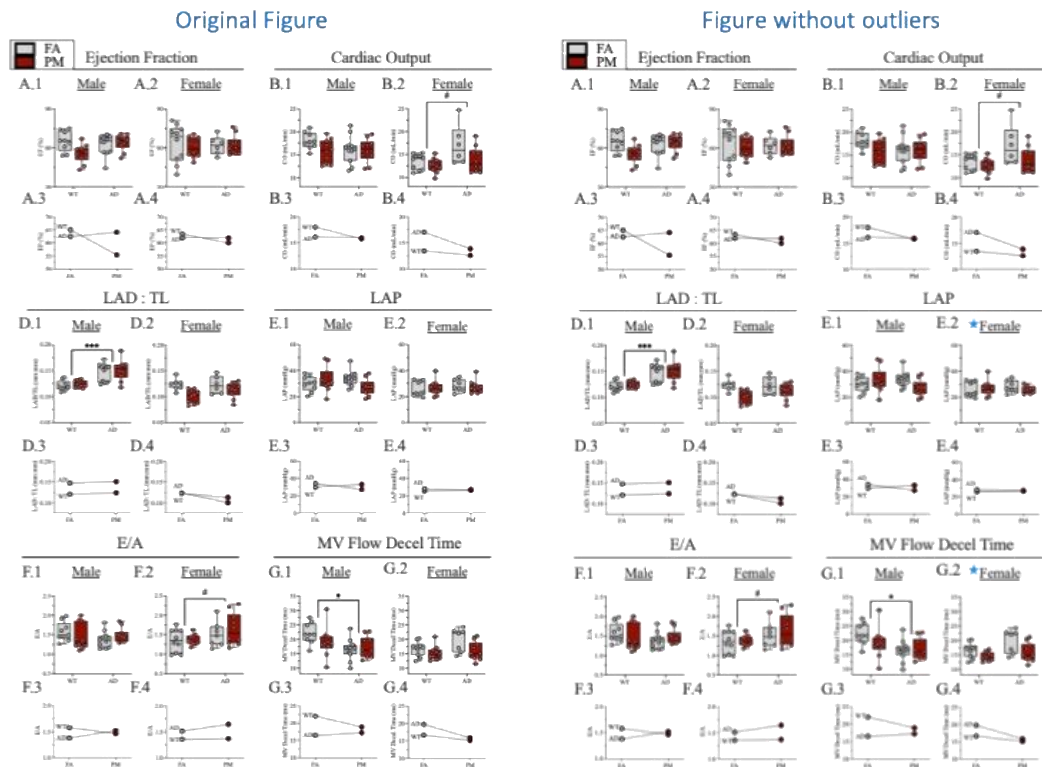


Figure 5

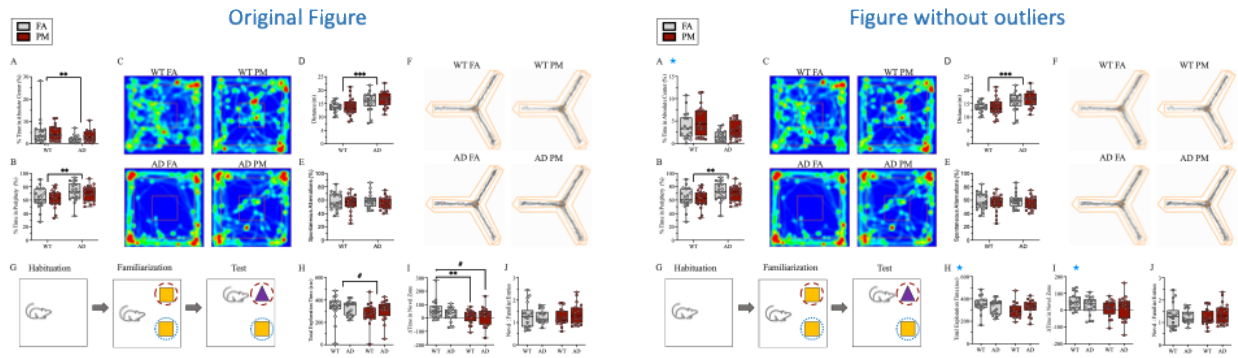


Figure Sup4

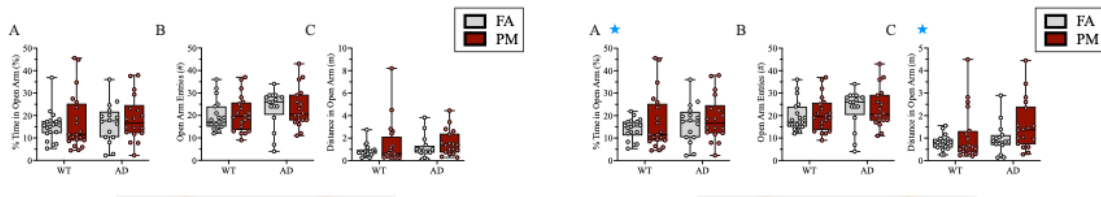
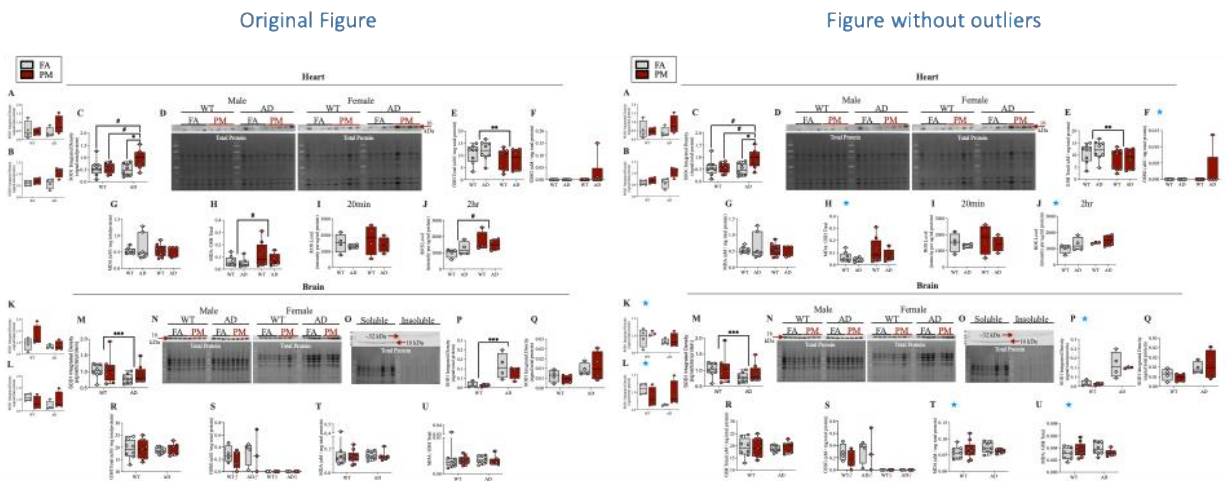


Figure 6



Examples of this reflect the following:

- 2) “Similar to the ELISA data, western blot analysis of male heart samples showed a trending increase in Ab42 level in AD mice relative to WT ($t(1, 13) = -1.99$, $p=0.068$) (Fig. 1D; S2A-B).” However, this reported trending increase is driven by a single mouse outlier, and other data in figure 1F have high variability driving the interpretation due to the small sample size explored. Further, the inability to report female data is a significant limitation of the study.

We thank the reviewer for specifying the data that are of concern. The reviewer is concerned that for ELISA and WB of male heart samples the result would be driven by 1 outlier. We were limited

in the WB by the number of samples that could be loaded for comparison in the same gel having 4 groups reducing power. For the ELISA data of Fig.1, outlier testing did not reveal outliers for both 1D and 1F. For the female mice, we have included the western blot (WB) membranes in the supplemental Fig S3. We were able to successfully get a signal for A β 42 in both the heart and brain, albeit only for 1-2 samples per membrane and we were not able to obtain a signal for A β 40 in either organ in female samples. The lack of signal may require a more sensitive method to detect amyloid beta in female samples at this early age possibly due to the low expression level. To increase both sensitivity and power (a concern for Rev.1) we utilized an ELISA kit. We have reordered the ELISA and WB figures and have further clarified the text as lack of clarity might have led to the reviewer's concern (Page 6, lines 6-15; Page 7 lines 16-20; Page 8, lines 12-15; Page 18, lines 9-13).

- 3) Similarly, this comment is driven by 1 or 2 outliers with the vast majority of samples in the WT range. "Instead, there was a trending increase in Ab42 specific oligomer signal (using VIA oligomer structural antibodies) in AD mouse hearts ($t(1, 29) = -1.92, p=0.064$) (Fig. 2B, S3)". It is also difficult to interpret the significant increase in total plaque in male brain samples when 60% of the samples have normal values (similar to WT males) and the other 40% are elevated. See figure 3A.

We agree with the reviewer's concern re the trending increase for the VIA oligomer's levels and we removed the symbol from Fig. 2. We restated the findings to better reflect the data presented in Fig. 2 (Page 8 lines 17-21; Page 9, lines 5-6, 8-10). We also added a paragraph with the interpretation of the integrated data on aggregation (Page 19, line 23 and Page 20, lines 1-9)

- 4) Once again it is also difficult to interpret the significance here when most of the sample is similar to FA and the increase is driven by 2 mice. "Although there was no effect of exposure on whole plaque growth (Fig. 3E-G), plaques that were smaller in size before exposure (less than 250 μ m²) grew more than plaques that were originally larger in size (greater than 1000 μ m²) ($t(1, 300) = -1.64, p<0.001$) (Fig. 3H)."

We apologize for the confusion in regard to the brain plaque data of Fig.3 as we tried to display the data of the complex integrations of two genotypes and two exposures across the different experiments resulting in different presentation of the data. We only included AD models exposed to FA or PM_{2.5} as WT mice do not display the stereotypical AD plaques at this early age. We have now specified in the figure that mice are AD (APP/ Δ E9). While there is a ~40% increase in plaque numbers in AD exposed male, the growth difference of plaques of different size at baseline we believe accounts for the finding as the plaque growth plateau has been described in the literature (as explained below). We believe that the data remains important in that it demonstrates that PM_{2.5} exposure increases the AD pathology over the AD genetics.

In regard to the difficulty to interpret significant increases in total plaque count. Our linear mixed model revealed a significant effect of PM_{2.5} exposure in Fig.3B-C. We retested the outliers on figure 3G that revealed a data point in the FA exposed mice (the highest point) to be an outlier. Excluding this data point would bolster the findings that PM_{2.5} exposure increases plaque growth,

as the difference between the two groups increased. However, like the rest of the datasets, we have included outliers to maintain rigor (see figure 3 above).

We apologize if the reviewer also found difficult to interpret the results of Fig.3H. There is no difference between FA and PM_{2.5} exposure within groups based on the initial size of the plaque. However, we found a significant effect of initial plaque size on their growth over the exposure period. Specifically, plaques that started at <250um² (smaller plaques) grew significantly more than plaques that started at a larger size. This result highlights another important discovery that there might be a cap in the size the plaques can reach so that larger plaques do not grow significantly larger.

We now tried to better explain the findings in Figure 3 in the text (Page 10 lines 1-4, 7-9; Page 19, lines 12-13, 16-19; Page 19, line 23 and Page 20, lines 1-9).

- 5) There are also concerns with the cognitive data. The group are making statements based on trends, which are driven by outliers as it comes to the cognitive changes in Fig 5. "PM2.5 exposure, rather than genotype, influenced the results of the Novel Object Recognition Test (Fig. 5G). PM2.5 exposed mice had a trending association of decreased total exploration of objects during the habituation phase of the assay (t (1, 69) = -1.71, p=0.092) (Fig. 5H)"

We acknowledge the reviewer's concern re the variability of the cognitive readouts. Such variability is known in the field so that the recommended power requires a larger number of mice than for cardiovascular function. Additionally, as reported in the literature, mouse models of AD often display significant changes in behavior at much later age, with earlier onset achieved by combining multiple human mutations in the mouse genotype with the 5X FAD beginning to display behavioral changes between 4 and 6 months of age. We are not surprised that the changes observed are not statistically significant, although we begin to observe changes by genotype and more with exposure. We tried to better explain the finding on Page 14 lines 1-5; Page 15, lines 16-17; Page 22, lines 3-4, 6-17.

- 6) Over statements are once again driven by outliers "Interestingly, a detectable level of oxidized glutathione (GSSG), although not significant, was observed only in the AD-PM2.5 mice but not in the other groups (Fig. 6F)." As such the conclusion that "This data suggests that in the heart, PM2.5 exposure leads to deleterious changes in redox homeostasis and antioxidant responses." reflect more of an over-embellishment of data interpretation. As such conclusions should be tempered down.

We apologize for the lack of clarity. While GSSG is one of the markers, often difficult to detect, other markers appear to be significantly changed. The levels of SOD are significantly increased in the heart of exposed AD mice and exposure of cardiomyocytes to plasma from PM_{2.5} mice significantly increases ROS production after 2 hrs. The conclusion sentence indicated by the reviewer refers to the interpretation of the overall results from the panel of markers tested.

- 7) Although the authors have run the required sex by group interactions and found in several cases where no sex differences were found, thus they grouped the male and female data.

However, given the variability in data, and the sex-reported data in other data, I would want to see the figures broken up by sex. Further, the figure legends need to show how many male and female samples are explored, currently, the author only reports the total sample size.

To keep the figures from becoming too busy, we have combined the male/female results when there were no sex differences (sex:genotype or sex:exposure). We have now included each figure broken up by sex in the supplementary data (Figures S4, S5, S7, S8-S10) and updated the results throughout (Page 5, Lines 4-8; Page 6, lines 10-15; Page 7, lines 16-20; Page 8, lines 20-21; Page 9, lines 5-6, 8-10; Page 12, lines 21-22; Page 13, lines 1-2, 9-10, 12, 15-16, 20-23; Page 14, lines 1-5; Page 15, lines 5-7, 8-9, 12-13; Page 16, lines 11-13; Page 24, lines 4-15) and figure legends to reflect the sample size for males and females, as suggested by the reviewer.

- 8) Given that the echo data were collected under light anesthesia it is important to report the heart rate for the mice as that may be the driver for the slight differences noted in CO between groups.

We apologize for the lack of clarity as the echocardiographic Table 2 is busy. Heart rate for the echocardiography data can be found in Table 2, first data row.

- 9) As the group is exploring the effects of PM_{2.5} at younger ages, it is important that in the discussion the authors reflect this, given that the AD model typically are indistinguishable from non-transgenic mice on cognitive tasks at six months, the authors should point out how their data reflects others at a young age etc. And if others have reported sex differences in the AD cognitive changes at that age.

We appreciate the recommendation of Rev.1 and Rev.2 to further discuss the importance of exploring the effects of PM_{2.5} at a younger age. We have further discussed cognitive outcomes with differing lengths and concentrations of PM exposure (Page 12, lines 16-17; Page 13, lines 16-17; Page 18, lines 12-13; Page 20, lines 7-9; Page 22, lines 16-17; Page 23, lines 16-18; Page 24, line 22; Page 25, lines 1-2).

Reviewer #2 (Remarks to the Author):

The relationship between cardiac failure and Alzheimer's disease (AD) is an interesting and active research area. A β deposition has been found in the heart of AD patients and patients with heart failure, which raised the term cardiac amyloidosis. AD often co-occurs with heart failure and the two diseases share some common risk factors. However, the association of poor heart health condition with AD and their interaction is rather complicated and can be modified by environmental factors independent of aging. This paper demonstrated that exposure to particulate matter (PM) may have an impact on both the heart and the brain amyloidosis. The manuscript has a clear emphasis on the effects of PM_{2.5} exposure on AD pathology in both transgenic AD mice and WT mice. The study was well designed with proper controls and clear data presentation. What is more interesting is the male vs female sex differences comparison in

both AD markers and cognitive functional evaluation. Overall, the conclusions were supported by the data provided in the manuscript. However, a few things can be improved to make the paper more convincing.

We appreciate Reviewer's 2 recognition of the significance of the study within the "interesting and active area of research", the emphasis the study has on the effects of PM_{2.5} exposure on AD and of the study well designed.

1. Although the author acknowledged the differences in the chemical composition of PM for male and female mice exposure, it was not clear how this huge variation was introduced. Were they exposed sequentially during different months of the year? Also, the timeline in Supplemental Fig. 1A is a little confusing. Looks like the exposure was just around 60 days or so in this graph but the authors stated in the paper that the PM exposure was 3 months long. Some clarification/correction seems necessary.

We thank Rev.2 for pointing out the clarity needed regarding our exposure model. Males and females were not exposed at the same time due to chamber availability, thus we have updated the text to reflect the time of year each sex was exposed. Further, while mice were exposed over a period of three months, this didn't include weekends and holidays where the mice were not put in chambers. We have updated the text to include the information (Page 5, lines 4-8).

2. The manuscript mentioned that a few AD-related phenotypes (such as hyperactivity and anxiety) were not changed post PM_{2.5} exposure. The authors believe that this is due to the young age of the mice (6 months old) evaluated in this study. It will be more convincing to provide some references using the same 2x Tg AD mouse model to back up this claim. Again, it might also be due to the relatively short PM_{2.5} exposure if the mice were only exposed for 2 months.

Similar to Rev.1, Rev.2 commented on the cognition and young AD mice. Please see the response to Rev.1 critique #5.

3. The analysis of sex differences was very interesting in this study, although the authors did not observe any sex differences in behavior and functional measurements. Sexual dimorphism in AD patients is very obvious, but these differences in animal models can be model specific with the 3xTg AD model being the most prominent one (Age and gender differences for the behavioral phenotypes of 3xTg Alzheimer's disease mice, Brain Research, Volume 1762, 2021; Dennison, Jessica L. et al. 'Sexual Dimorphism in the 3xTg-AD Mouse Model and Its Impact on Pre-Clinical Research'. 1 Jan. 2021 : 41 – 52.). Some expanded discussion on this topic might be interesting to certain readers.

We thank the reviewer for finding the analysis of sex differences interesting. We acknowledge Rev.1-2's critique on expanding more on the topic throughout the manuscript. As mentioned previously, we did not discuss the sexes separately when there was not an interaction with sex and another factor (i.e. genotype and exposure) because any changes within the data were going

in the same direction (Page 12, lines 19-22). However, we have now updated the results of each dataset to include any differences between sexes (Page 5, Lines 4-8; Page 6, lines 10-15; Page 7, lines 16-20; Page 8, lines 20-21; Page 9, lines 5-6, 8-10; Page 12, lines 21-22; Page 13, lines 1-2, 9-10, 12, 15-16, 20-23; Page 14, lines 1-5; Page 15, lines 5-7, 8-9, 12-13; Page 16, lines 11-13). We have also expanded our discussion on sex differences in cognition as requested by Rev.2 (Page 24, lines 4-15).

4. The running title might be more appropriate by replacing Cardiac Alzheimer's by Cardiac Amyloidosis

We understand Rev.2's recommendation to change the running title from Cardiac Alzheimer's to Cardiac Amyloidosis. However, this would raise confusion among clinical cardiology who often recognizes cardiac amyloidosis as that of light chain and TTR with extracellular aggregates, a paradigm that the recent discoveries have ousted, yet maintaining its momentum. Further our study tests the cardiac pathological and functional changes in a mouse model of AD. We therefore specified AD 'pathology' in the title.

Reviewer #3 (Remarks to the Author):

The manuscripts focused on the associate of PM2.5 and heart failure/AD, which is important for those researchers in such field. It is interesting and meaningful. The manuscripts might be considered to be accepted after some revision.

We are thankful to Reviewer 3 who found our study important for the field, interesting and meaningful and who considered the study acceptable pending the suggested revisions.

1. The components of PM2.5 play an important role on their toxicity, the author should give some discussion on the components.

We appreciate the feedback from Rev.3 regarding the toxicity of the components of PM_{2.5}. We have strengthened the discussion on this topic (Page 23, lines 20-23. Page 24, lines1-3). Further, per Rev.3's request, we have highlighted our introductory statement about the ability of PM_{2.5} to translocate to other organs (Page 5, lines 4-8).

2. Considering the PM2.5 was more easily enter into lung, the author proved that the PM2.5 was associated with the AD and heart failure, is there any evidence that PM2.5 could enter into brain or heart?

We agree with the reviewer on the importance of testing the potential direct effect of PM_{2.5} to the heart and brain. We included this aspect and the related references in the introduction (Page 4, lines 1-4).

3. Would the biochemical indicators of the blood be affected by the PM2.5?

We thank Rev.3 for suggesting looking at blood biochemical indicators. Metanalysis studies in humans have documented changes in the lipid panel, metabolic hormones (insulin), cell and humoral inflammatory markers and coagulation factors. We run the biochemistry panel using the Zoetis VetScan VS2 on the available samples from male and female from all groups which would have provided additional information, void of the confounders of the individual's variability of the human subjects published data. Unfortunately, due to the freeze-thaw of the plasma samples leftover from the already performed experiments we didn't have enough freshly frozen samples left. Therefore, upon analysis, the values were lower than the normal ones across many parameters. We also obtained new samples from the group in Ohio (although from a different cohort) but we later realized that the collection of the blood in heparin affects several chemistry values. We therefore do not feel confident in including the data in the manuscript although the results showed no difference across the groups. We added this important consideration in the limitations paragraph (Page 26, lines 9-13).

4. Did the structure of the brain affected by the PM_{2.5}? the author may provide some brain tissue slices.

We thank the reviewer for this insightful comment. Unfortunately, here too, tissue is not available to fully address this concern, albeit valid. Accurate evaluation of brain changes would require whole-brain assessment of overall structure and DTI tractography analyses to assess PM_{2.5} effects on axonal tracing. While we do have sectioned brains from treated animals remaining, they have already been sliced and mounted in a manner that is not conducive for large-scale structural analysis of gray and white matter changes. The assessment of structural changes can be a subject of future studies, which we have added in the limitations paragraph (Page 26, lines 6-8).

5. Why the author only evaluates three months effects on mice, not more longer time?

We agree with Rev. 3 that longer exposure would provide statistically more significant data based on the results we obtained. We started with the 3-month exposure based on the available literature that considers 3 month a chronic exposure. This study being limited to three-months PM_{2.5} exposure allowed us to study early disease progression to determine key time points for intervention. However, we agree with Rev.3's and a comprehensive analysis across longer periods of exposure (i.e. 6 months) is planned for future studies. We now discussed this aspect in the limitations paragraph (Page 25, lines 16-20).

We would like to thank the reviewers for their positive evaluation of the revised submission. The manuscript has notably improved thanks to the reviewers' comments. Changes are with blue fonts

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have done well to address my concerns.

We would like to thank the reviewer for the positive comment and helping us improving our manuscript.

Reviewer #2 (Remarks to the Author):

This work by Butler et al concerns the complex interactions of genetics x environmental factors in the condition of a cardiac Alzheimer's disease, and the topic of research is important and interesting. While mainly focusing on the early stage or a relative shorter period of exposure to airborne particulate matter, the authors did demonstrate air pollutants as one of potential risk factors to the development or progression of heart and brain proteinopathy. The revised manuscript has greatly improved data accuracy as well as the clarity in communication. I'm pleased that the authors have addressed all my concerns and suggestions.

We would like to thank the reviewer for the positive comment and helping us improving our manuscript.

Reviewer #3 (Remarks to the Author):

The present study focuses on the effects of particulate matter towards heart and brain, which is meaningful and interesting for the full understanding of PM on human health. In comparison with previous versions, the revised manuscript has been improved significantly. The data in the manuscript could support its conclusion. Moreover, the manuscript has been carefully revised according to the reviewers' suggestion. However, there is still some issues that should be addressed.

In the manuscript, some discussion on the entrance of PM into brain should be added. In my opinion, the manuscript could be accepted after the revision.

We would like to thank the reviewer for the positive comment and helping us improving our manuscript. We discussed the direct effect of PM entrance into brain (Page 3, Line 23; Page 4, Lines 1-2 and 4-5; Page 22, Lines 21-23; Page 23, Lines 1-6).