Author’s response to reviews

Title: Effects of Homocysteine on White Matter Diffusion Parameters in Alzheimer's Disease

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Dear Professor Sabbagh,

Thank you for allowing us to revise our original manuscript entitled “Effects of Homocysteine on White Matter Diffusion Parameters in Alzheimer’s Disease”. We appreciate the suggestions and comments from you and the reviewers. These suggestions and comments formed the basis for most of the changes we made, all of which are marked in red text.

In this revision, we have attempted to clarify any issues raised and to make the manuscript more concise. Please find our point-by-point responses to the suggestions and comments below, all of which are adequately reflected in the revised manuscript.

We hope that the editorial board will agree that this study will be of particular interest to your readers. We look forward to hearing from you.
Reviewer reports:

Barbara B. Bendlin (Reviewer 1): Additional questions and comments are as follows:

1. The introduction discusses white matter hyperintensities (WMH) and the investigators suggest that they will examine WMH in this study (as stated on pg. five: "we hypothesized that hyperhomocysteinemia in late-onset AD may lead to a greater WMH loads"). However, WMH are typically evaluated using T2FLAIR weighted imaging (or T2 and PD), not DTI. In this study, the investigators are examining white matter microstructure; this should be corrected.

Response: We thank the reviewer for this and all of their suggestions. The WMHs related to higher homocysteine may reflect changes in diffusion tensor parameters. Therefore, we used these parameters to correlate with the clinical outcomes. To make the sentence clear, we revised the sentences (page 5, line 4 and line 8).

2. The investigators conclude that "homocysteine levels reflected renal dysfunction status and decreased vitamin B12 and folate state that required clinical attention as they may associated with impaired WM microstructural integrity and modulate the cognitive performance in cross sectional observation". I do not believe this is shown in the current study. There were no main effects or interaction effects of homocysteine on cognitive function. Again, in the discussion section, the paper states that "Randomized, controlled and longitudinal follow-up studies are warranted to clarify whether the homocysteine lowering treatment can improve
the cognitive outcomes in the future”. In the current study, there does not appear to be an effect of homocysteine on cognitive function.

Responses: Based on our results, a direct relationship between homocysteine and cognitive outcome was not significant. However, homocysteine may interfere with white matter integrity, and white matter integrity may predict cognitive measures. We found that several factors may modulate the relationships between homocysteine and white matter, including eGFR, creatinine, folate and vitamin B12 levels. These results formed the basis of the conclusion that "homocysteine levels reflected renal dysfunction status and decreased vitamin B12 and folate state that required clinical attention as they may associated with impaired WM microstructural integrity and modulate the cognitive performance in cross sectional observation". To avoid confusion, we have revised the conclusion (page 17, line 20-23).

3. Were any data lost or excluded? In a study of AD, it would not be uncommon that some participant data are excluded due to motion or other challenges in scanning this population.

Responses: Based on our study scheme, patients were included after the consensus of a panel composed of neurologists, neuropsychologists, neuroradiologists and experts in nuclear medicine. To comply with the International Working Group criteria for the diagnosis of AD, all 132 subjects in this study already had a baseline MRI scan that was sufficient to judge whether they could tolerate the research protocols of diffusion tensor and 3DT1 weighted imaging. The acquisition time for these images was less than 20 minutes. As our patients all had mild AD, they all tolerated the procedure.

4. APOE4 genotype was determined but not included in the analysis. Prior studies suggest APOE4 genotype may be associated with homocysteine levels, this may be a factor to consider here.

Response: We analyzed differences between E4 and non-E4 carriers and the homocysteine levels were not significantly different. (page 10, line 9-10)

5. Can the authors provide their rational for conducting both a TBSS analysis, and ROI analysis (i.e. tractography analysis)? The analyses use the same underlying data, so it may be sufficient to present just one set of analyses.

Response: TBSS allows for more specific delineation of anatomical changes between group differences or correlations with biomarkers. For tractography, we treated each fiber bundle as an independent parameter. Specific to this research protocol, the TBSS results were taken to reflect
regional changes whereas tractography results were taken to reflect structural connection alterations. As they reflect complementary information, we included both results in this study. (page 8, line 5-11)

6. The statistics section indicates that SPSS was used for all statistical analysis. This should be clarified to indicate that FSL was used for voxel-wise analysis.

Response: To avoid confusion between clinical/laboratory data analysis and neuroimaging analysis, we have revised the section of statistical analysis as suggested (page 9, line 17-19).

7. Given that FA, MD, RD, and AD were assessed in 11 ROIs, and used to predict several cognitive scores, and as well as tested for associations with several lab values, should the authors perhaps consider correcting the ROI analyses for multiple comparisons as well?

Response: In theory, statistical analysis using corrections for multiple comparisons allows for more stringent post-hoc testing. For the p values of the 11 fiber bundles using Bonferroni’s correction, the p value needed to be less than 0.001 (0.05/11/4 = 0.0011) and would increase the false negative rate. As the 11 ROIs were not independent covariates and to balance type I and type II errors, the significance in this study was defined as a p value <0.01. We have changed our results accordingly (page 9 line 19-21).

8. What was the purpose of conducting the analyses with and without covariates (confounders), for example, as presented in pages 10-11? Was the idea to determine whether these factors mediate the relationship between homocysteine and white matter microstructure? The motivation for various analyses is not adequately described, and the results not fully discussed.

Response: Based on the correlation analysis, homocysteine levels were correlated with eGFR, creatinine, folate and vitamin B12 levels. The rationale for adding these confounders was to understand whether the impact of homocysteine on the diffusion parameters was independent or modulated by these factors. Our results suggested that the significance of homocysteine on the WM parameters may have disappeared after controlling for these factors. Therefore, the impact of homocysteine on the WM microstructure was not fully independent. We have revised the results section as suggested (page 10, line 24-25 and page 11, line 1-2).

For the correlation analysis between WM integrities and cognitive measures, we further adjusted for homocysteine and related biomarkers to understand whether the impact of WM integrities on cognitive function was mainly through homocysteine and its related factors. The results showed
that most significance still persisted even after adjusting for the factors, suggesting that changes in the WM microstructure were independent of homocysteine with regards to the influence on cognitive performance. (page 11, line 10-13).

In the discussion section (subtitled Homocysteine level was not directly related to cognitive impairment, page 14, line 24), we have highlighted the relationships between homocysteine, white matter microstructural changes and cognition.

9. The first paragraph of the abstract is not clear and could be improved.
Response: The first paragraph has been revised (page 2, line 2-8).

10. The conclusion that the findings are consistent with data on the methylenetetrahydrofolatereductase gene should be explained.
Response: Higher levels of homocysteine have been reported in the T variants of the methylenetetrahydrofolatereductase gene. However, the cognitive outcomes were not associated with the risk genotype group, suggesting that the clinical significance of cognitive outcomes may be considered as minor. We found an association between hyperhomocysteinemia and WM integrity, however the direct association between hyperhomocysteinemia and cognitive outcomes was modulated by other factors as discussed in the paragraph “Factors that may modulate the impact of homocysteine”. Therefore, we concluded that the findings between hyperhomocysteinemia and cognitive outcomes were consistent with the methylenetetrahydrofolatereductase genetic data. We have clarified the sentences in the revised manuscript accordingly (page 15, line 22-25).

11. Grammatical errors need to be addressed throughout the manuscript.
Response: The manuscript has been edited for English.

Minor:
1. The authors might consider leaving out the word "different" in the title.
Response: We have revised the title accordingly. Thank you.
Pradip Kumar Umar Kamat (Reviewer 2): The manuscript by Lee et al entitled "Effects of homocysteine on different White Matter diffusion parameters in Alzheimer's Disease" is well designed and significantly relevance for understanding of AD pathogenesis, As per clinical point of view manuscript is well presented. Only I can see that plasma concentration of homocysteine greater than 14 μM increases the risk of Alzheimer disease (AD). While the investigator only found the level 12.7 ± 4.4 μM which is not so upregulated in observed AD patients (data Table 1). Authors also found that Homocysteine level not relate directly to cognitive impairment in their study may be because of not sufficient upregulation of plasma homocysteine level. Hence how the study established the effects of homocysteine on different White Matter diffusion parameters in Alzheimer's Disease is not making clear interpretation.

Response: The authors thank the reviewer for the comments. As the reviewer pointed out, most of the patients had levels of homocysteine of 12.7 ± 4.4 μM. Because the population in this study was composed only of patients with AD, our study design was not able to investigate whether hyperhomocysteinemia is a risk biomarker for developing AD. Rather, this study explores whether homocysteine may be considered as a symptom modulation biomarker, rather than a diagnostic marker for AD.

Our results showed that homocysteine levels may modulate the white matter integrity that determines cognitive outcomes. However, a direct relationship between homocysteine and cognitive outcomes was not established. Whether a wider range of homocysteine levels may help to establish a primary relationship between homocysteine and cognitive outcomes requires further studies. Thank you for the suggestion, we have included it in the study limitations (page 17, line 2-10).