nature portfolio

Peer Review File

Adhesion molecules provide an endothelial protein signature in preclinical and clinical Alzheimer's disease.

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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)
Summary:

This study utilized CSF and plasma-targeted proteomics (276 proteins) from 354 Brain Stress Hypertension and Aging Program (BSHARP) participants. The 276 measured proteins clustered into five modules associated with CSF Amyloidβ42, Tau, and pTau. (all p-values <0.05). A CSF protein AD signature was characterized by elevated levels of CSF Hepatocyte Growth Factor (HGF), Intercellular and Vascular Cell Adhesion Molecule 1 (ICAM-1, VCAM-1), Neuropilin 1 and 2 (NRP-1, NRP-2), Scavenger Receptor Class B Member 2(SCARB2), Plasminogen Activator, Urokinase (PLAU). (all <0.05). Moreover, this study suggests a proteomic signature in the CSF of individuals with preclinical AD that is driven by adhesion molecules and might be implicated in the pathogenesis of AD. While this study is
interesting and technically sound, the data is not convincing enough to support the conclusions. The specific points are listed below-

Major comments-

1. This study utilized both CSF and blood plasma to evaluate AD biomarkers. Further, they reported the CSF outcomes of AD biomarkers in Table 1 but didn't discuss plasma proteomic levels of ABeta, tau, pTAU, etc. The authors should include these datasets in Table 1 or supplementary figures. Also, the author(s) should comment on which forms of pTAU epitopes they have been studying, as different forms of pTAU have been linked to AD disease progression.
2. Figure 3 A-C; panel B, Tau correlation with VCAM1 is missing. Kindly add this to the figure. Also, the correlation coefficient of each critical protein in individual figures (3 A-C) should be mentioned.
3. Figure 4B and 4C: In the result section (sentence 195-201), the author has commented that "We found that six of the critical proteins identified for both cognitive function and TAR status (HGF, ICAM1, VCAM1, NRP1, NRP2, SCARB2, PLAU) were differentially expressed between the four groups (all had a of P<0.05)…". However, the authors should describe the figures well and highlight the dataset comparisons between the four study groups (normal cognition (NC-), preclinical AD(NC+), prodromal AD(MCI+), and participants with non-AD MCI (MCI-). Also, the author(s) should focus on the statistical differences between normal cognition (NC-) vs prodromal AD(MCI+) and preclinical AD(NC+) vs prodromal AD(MCI+). Further, the mean (SD) values should be written in the result section test. It will help readers understand the protein level differences across the group. Showing only a p-value won't be sufficient.
4. Despite the original datasets, there is an opportunity for significant improvement –
a) This paper requires major rewriting in the introduction, methods, and discussion sections, as many sentences remain unclear because of grammatical errors or disorganized sentence frames. The introduction section is also small and vague.
b) The author(s) have identified novel biomarkers based on the datasets. However, in summary, the author(s) have claimed that their findings are linked to molecular mechanisms and therapeutic targets. The authors should provide explanations and discuss this in the publication.
c) Since this paper is about establishing biomarkers for AD, the author(s) should elaborate more on the previous research linked to AD biomarker establishments in the introduction and discussion sections.

Minor comments:

1. In sentence 127, the authors mentioned, "The Hub proteins were defined as the top 10% of proteins associated with clinical traits and have the most connectivity to other proteins in each module…". The authors should define which clinical traits were utilized for this consideration.
2. In Table 2 and Figure S2, the author has defined progressor and non-progressor groups. The author should report the basis of the classification

Reviewer #2

(Remarks to the Author)
This is an interesting study using the Olink platform to find proteomic signatures in Preclinical and clincial AD. The study design seems sensible and the narrative is clear. The impact of the paper relies on the analysis of the paper being solid but there are key details missing to be fully confident. Thus, I have some concerns which should be addressable by the authors.

1) The authors lean heavily on $P < 0.05$, but do not mention effect sizes. Given the large sample sizes it would be useful to characterise these effects rather than just p-values.

2) Are the statistical analysis adjusted for multiple hypothesis testing?

3) Are the statistical analysis adjusted for the population covariate e.g. age, sex etc.

4) Without access to the code to perform the statistical analysis, it hard to verify whether what the authors think they did aligns with what they actually did

5) Its quite hard to read a lot of the figure axis and labels

6) Figure legends need to be improved. For example, Figure 3 - this doesnt really explain the plot at all. Figure 2. what do the lines and colours mean on the PPI plots mean?

7) Some of the correlation looks like they could be driven by individual points, and I'm not sure if these are individual people from the current explainations. In any case - are these correlations robust to some of these more extreme measuremnets?

Reviewer #3

(Remarks to the Author)
The paper has an original and relevant question, namely how vascular function biomarker would contribute to AD progression. However, the design is not logically addressing this question, and mostly shows pathways analyses and correlations. For the question, it would be important to start with protein expression across the different stages of AD.

The paper lacks crucial details in the methods section. For example, the diagnostic criteria for defining MCI or AD are not articulated.
Moreover, the Simoa kit is not articulated: was it the N4PE kit?

It is not clear whether this is a population based study? This is not clear from the introduction and methods.
For the Olink analyses: were data below the LOD included in the statistical analyses?
Only few (two) adhesion molecules were measured in HABS, which makes it not such a strong validation cohort. Thus, validation of the majority of findings is essentially lacking and weakens the impact of the study, especially of its proteomics design and findings.

The number of patients included varies from 375 in the methods section to 354 in the results section.

A very strong element is the large number of non-white participants (41%). Recent studies showed biomarker differences between African Americans and white Americans, and prevalence differences of AD among demented patients. it is relevant to explore the influence of ethnicity on the biomarker results (thus not only ethnic differences being reflected in the biomarkers, but how ethnicity affected other observations), and include that in the aim.

Essential results are lacking, i.e. how many proteins were differentially regulated across the different groups? This information is lacking from the narrative. The authors dive immediately into the hub proteins, and the results miss details and specifics to really understand the findings. Correlations with AD proteins seem to be the goal, but this is not so informative and it is unclear what question this answers as main result.

The paper switches the focus from CSF to plasma, making it unclear what exactly is being replicated.

Apparently the plasma markers as add-ons and measured by Simoa and not on PEA, are replicated in the HABS cohort. This is out of focus of the title and main aims, and is really weak.

There is a lack of reference in the discussion. E.g (but more often, check carefully): -all known contributors to the pathophysiology of AD. Reference?

Table 1, unit for Olink proteins is lacking. Why only mention those 3 proteins?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The author has carefully addressed the reviewer's comments and incorporated the detailed requirements, which have substantially improved the overall quality of the manuscript. However, a few points still require further clarification, and I have outlined my comments below for consideration.

1. In the 3.1 Sample characteristic section, the number of cases of each group (outlined in the text) does not match the numbers shown in Table 1. Kindly review it and make sure that these numbers match. Otherwise, this can create confusion for the readers.

2. The authors should rewrite the 3.1 Sample characteristic section of the results. The table has so much information, but the authors have not described anything in the text. Kindly review it and describe other measures in results, including Neuroimaging, cognitive assessments, etc.

3. In section 3.3 of the results, the author should give the mean difference values with p-values. The p-value alone does not define the effect size. It is essential to denote this in the main text here.

4. The author has not mentioned anything about the existing biomarkers in the discussion section. Recently, pTau 217 has been established and validated by many groups as a blood-based biomarker. Also, GFAP is being consistently studied as a reliable measure for AD pathology. The author (s) should discuss the above suggestions in the discussion section.

5. In Figure S5, which statistical test was used to compare the CSF hub protein levels? The authors should provide the details in the figure legend.

Reviewer #2

(Remarks to the Author)
I thank the authors for revising their manuscript - many aspects have been improved. However, I still think the figure and table legends need to be improved to make them self-contained. For example, boxplots should describe what exactly is meant by the central line - as these differ between implementations. Futhermore, in figure 3 the legend still does not describe what the colour of the node or lines mean clearly in the legend. Why are some protein purple and some blue or green? Please go through each figure carefully making sure each detail is clearly explained in the legend.

Some information is given later in the manuscript (though in my experience it is much better for figures to be self-contained). I do not really undestand how I am meant to intepret these plots. What is meant by gene neighbourhood and gene fusion? What relevance do they have to manuscript? Why do I care if the structure is known or not? The authors are showing a lot of information in some of these plots and I'm not sure it's relevent to analysis. In the very least it is never explained in the manuscript.

why is every boxplot a different style?

The authors have now included effect sizes - though they dont explain in the table what these are. Some of these effect sizes are quite small. How are we meant to interpret these effect sizes in the wider context of the work?

I found the discussion didn't fully acknowledge the small effect sizes, the small size of the replication study, the risk of false positives and negatives and suggesting their study highlights new mechanism. I think the authors' data is valuable but it observational not mechanistic. To improve the discussion, it would benefit from being contextualised better in its limitations. The authors only get to this at the end so the limitations are not explicitly linked to each conclusion and implication directly. Rather they simply state the limitation of the work as a whole so it is disconnected.

Reviewer #3

(Remarks to the Author)
I appreciate the effort into improving the manuscript, and adding a real validation cohort.

For the abstract, it would be good add more numbers: such as the n of validation cohort, n per clinical stage, and main fold changes (or their ranges).
The last sentence of the abstract now reads as follows, and is therefore not correct: the signature in CSF plays a role in AD pathogenesis.

Important recent studies that analysed CSF proteomics of larger cohorts of AD patients, and preclinical AD by Olink PEA are not discussed and should be part of the introduction and the current results should be discussed in relation to these findings.
- The novelty and added value of the current work is therefore not clear.

The paper still needs some correction for grammar (e.g lines 174, 175, 183, proteins instead of protein). AND: only one hub protein (Tumor Necrosis Factor Receptor
181 Superfamily 12A (TNFRSF12A)) which has significantly lower expression in NC compared to MCI (P=0.0004),
182 irrespective of the biomarker's status.
Of note: this should have been easily have been noticed by the co-authors .

The comparison of ethnicities for CSF markeres is interesting. This should be done for plasma markers too.
section 3.6: repeat the total n of 36 in this results section.

Still, there is a lack of references after statements in the discussion. For example: 222 pICAM-1 has been shown to regulate microglial responses and is correlated with cognitive manifestations in AD, suggesting a dual role in both disease pathogenesis and clinical progression. Reference?

Moreover, it is identified as a
248 mediator of the inflammatory response of the endothelial cell dysfunction to support BBB. Reference?

Same in these two sentences:
Research indicates that levels of VCAM-1 and ICAM-1 are often higher among AA 274 individuals with AD, which may reflect a complex interplay of genetic factors, the prevalence of comorbid vascular 275 conditions, and socio-environmental influences. Furthermore, markers associated with cerebrovascular injury, such as 276 HGF and SCARB2, may be more significantly upregulated in AA populations, possibly indicating either a compensatory 277 neurovascular response or increased endothelial stress.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The quality of the manuscript has substantially improved, and newly added datasets strongly support the findings. Despite this improvement and for making the final publication, I have a few suggestions that would be important to address -

1. Section 3.2. For race, both CSF proteins and plasma protein levels were measured and compared (Figures 2E and 2F). However, only CSF protein levels are measured for gender (Figure 2C). The authors should also analyze plasma levels of ICAM-1 and VCAM-1 to examine gender effects.

2. In my previous review, I commented on the need to incorporate the statistical test used in Figure S5 to compare CSF hub protein levels. In this resubmission, the authors have abandoned the previous validation cohort due to a small sample size. In the new figure, the authors present results from a new validation cohort and describe the statistical tests used. This new result shows a correlation between ICAM (panels A, D, G), NCAM1 (panels B, E, H), and VCAM1 (panels C, F, I) with Abeta-42, Tau, and pTtau-181, respectively. Although the dataset is informative and shows a significant correlation, a few questions remain unexplored.

A) Were there any gender or race effects on the CSF protein levels of ICAM, NCAM1, or VCAM1? The authors should run these analyses (similar to Figures 2C and 2E) using this new validation group.

B) Were there any significant differences between the CSF protein levels of ICAM, NCAM1, or VCAM1 across different groups (NC-, NC+, MCI-, MCI+)? The authors should run these analyses and add a table similar to Table 3 using this new validation group.

Minor comments:

1. It can be informative to provide a small demographic table for the new validation group in the supplemental figure.

Reviewer #2

(Remarks to the Author)
The reviewers have addressed my concerns about the manuscript.

Reviewer #3

(Remarks to the Author)
The authors have made a good job in improving the discussion, citing more references, and addressing my other remarks. This is a well performed study.
A few small issues remain:

- end of the discussion: "In summary our study has identified l protein biomarkers'. More than one biomarker was identified.
- references: 34 is the same as 35.
- The authors should also discuss a recent Olink-based study in CSF of preclinical AD, namely that of Del Campo et al in Mol Neurodegeneration, CSF proteins of inflammation, proteolysis and lipid transport define preclinical AD and progression to AD dementia in cognitively unimpaired individuals.
Del Campo M, Quesada C, Vermunt L, Peeters CFW, Hok-A-Hin YS, Trieu C, Braber AD, Verberk IMW, Visser PJ, Tijms BM, van der Flier WM, Teunissen CE.
Mol Neurodegener. 2024 Nov 11;19(1):82. doi: 10.1186/s13024-024-00767-z.
PMID: 39523360
-

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The quality of the manuscript has substantially improved, and newly added datasets support the findings. I have no further comments.

August 13, 2025

Dear Editor:

We thank the reviewers for their excellent input. We have extensively revised our paper, including a new validation sample, and provided additional analyses and results as requested. In our revisions we removed the prior validation data that was deemed weak by the reviewers and now include a more appropriate validation sample from the same institution as the main data used for this manuscript provided by Dr. Erik Johnson and Eric Dammer from Emory. We also reorganized the author order based on the contribution and authorship guidelines. Below is a summary of these changes.

Reviewer #1 (Remarks to the Author):

Major comments-

1. "***This study utilized both CSF and blood plasma to evaluate AD biomarkers. Further, they reported the CSF outcomes of AD biomarkers in Table 1 but didn't discuss plasma proteomic levels of ABeta, tau, pTAU, etc. The authors should include these datasets in Table 1 or supplementary figures. Also, the author(s) should comment on which forms of pTAU epitopes they have been studying, as different forms of pTAU have been linked to AD disease progression***."

Response: We apologize for the confusion in our last version. To clarify we only analyzed CSF AD biomarkers but analyzed both CSF and plasma OLINK PEA proteins. We also clarify that we measured $pTau_{181}$ in this study. We provide the baseline characteristics of the sample divided by 4 groups based on their MCI status and CSF AD biomarkers in the revised Table 1. We also provide the concentrations of Ab42, Total Tau, $pTau_{181}$, and the Total Tau to AB42 ratio in the 4 groups. These are provided in **Table 1**.

2. "***Figure 3 A-C; panel B, Tau correlation with VCAM1 is missing. Kindly add this to the figure. Also, the correlation coefficient of each critical protein in individual figures (3 A-C) should be mentioned***."

Response: We revised Figure 3 to include VCAM1 and correlation coefficients were added to the figure.

3. "***Figure 4B and 4C: In the result section (sentence 195-201), the author has commented that 'We found that six of the critical proteins identified for both cognitive function and TAR status (HGF, ICAM1, VCAM1, NRP1, NRP2, SCARB2, PLAU) were differentially expressed between the four groups (all had a of P<0.05)…". However, the authors should describe the figures well and highlight the dataset comparisons between the four study groups (normal cognition (NC-), preclinical AD(NC+), prodromal AD(MCI+), and participants with non-AD MCI (MCI-). Also, the author(s) should focus on the statistical differences between normal cognition (NC-) vs prodromal AD(MCI+) and preclinical AD(NC+) vs prodromal AD(MCI+). Further, the mean (SD) values should be written in the result section test. It will help readers understand the protein level differences across the group. Showing only a p-value won't be sufficient.***

Response: Figures 4B and C are changed as requested and pairwise comparisons are added to provide statistical comparisons between the various groups. We also add **Table 3** in the results section presenting mean (SD), effect size, and CI.

4. "***Despite the original datasets, there is an opportunity for significant improvement –This paper requires major rewriting in the introduction, methods, and discussion sections, as many sentences remain unclear because of grammatical errors or disorganized sentence frames. The introduction section is also small and vague***."

Response: We have extensively revised this paper to address the prior weaknesses in the language and rigor of the various sections (introduction, methods, discussion, and abstract).

5. "***The author(s) have identified novel biomarkers based on the datasets. However, in summary, the author(s) have claimed that their findings are linked to molecular mechanisms and therapeutic targets. The authors should provide explanations and discuss this in the publication***."

Response: We clarify that this study only describes the differential protein expressions in the various groups of cognitive and AD biomarker statuses. Our molecular description is to provide a biological explanation for these differences. We removed the implication about therapeutic targets and only described that in the context of future research. Our study is not powered or designed to assess targets of AD therapies.

Minor comments:

1. "***In sentence 127, the authors mentioned, "The Hub proteins were defined as the top 10% of proteins associated with clinical traits and have the most connectivity to other proteins in each module…". The authors should define which clinical traits were utilized for this consideration***."

Response: For clarification, in Section 2.2 lines 97-98 were added to explicitly state which clinical traits were added in the final analysis. The lines read as follows "In the final analysis, we considered several crucial clinical traits: age, race, gender, cognitive status, and key AD biomarkers (Aβ42, tau, and p-Tau181, TAR) to identify protein network. This is now provided under section 2.6

2. "***In Table 2 and Figure S2, the author has defined progressor and non-progressor groups. The author should report the basis of the classification***"

Response: For clarification, we have added following section on progressor and non-progressor groups (Section 2.2). We used the follow-up data from B-SHARP to further classify participants into progressors vs non progressors using the change in the CDR-Sum of Boxes (CDR-SOB) over the duration of the study. Progressors were defined as those who had an increase in the CDR-SOB vs non progressors as those who had either no change or decline in their CDR-SOB.

Reviewer #2 (Remarks to the Author):

1. "***The authors lean heavily on P < 0.05, but do not mention effect sizes. Given the large sample sizes, it would be useful to characterize these effects rather than just p-values***."

Response: Thank you for your suggestion. We added a separate Table 3 with effect sizes

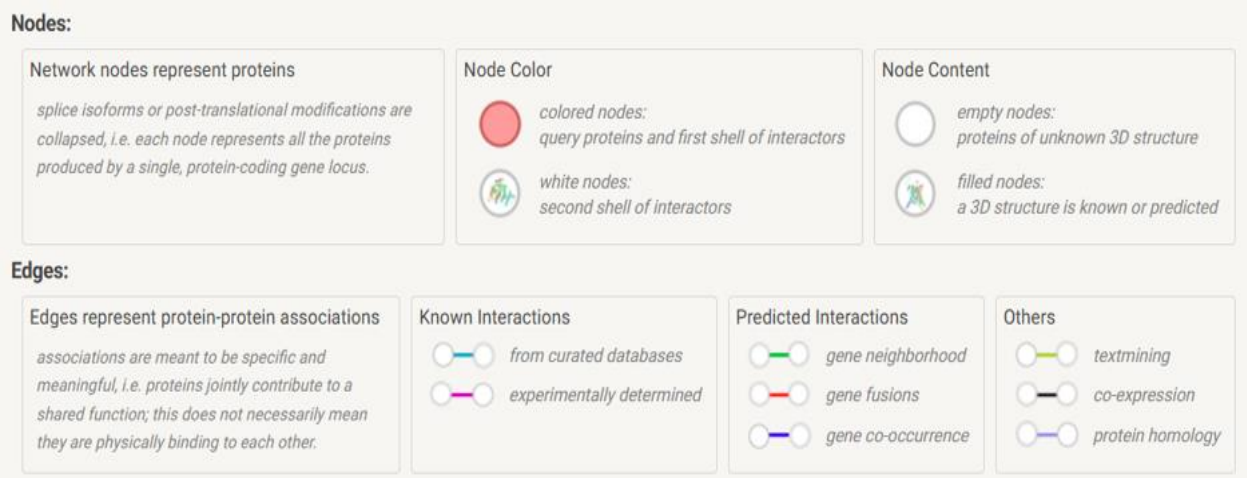
2. "***Are the statistical analysis adjusted for multiple hypothesis testing? Are the statistical analysis adjusted for the population covariate e.g. age, sex etc***".
Response: We have presented these results in the supplement (Table S.2) with false discovery rate corrections and adjustment for age, sex, and race.

3. "***Without access to the code to perform the statistical analysis, it hard to verify whether what the authors think they did align with what they did?***"

Response: The Code files are available as attachment

4. "***Its quite hard to read a lot of the figure axis and labels and Figure legends need to be improved. For example, Figure 3 - this doesnt really explain the plot at all. Figure 2. what do the lines and colours mean on the PPI plots mean?***"

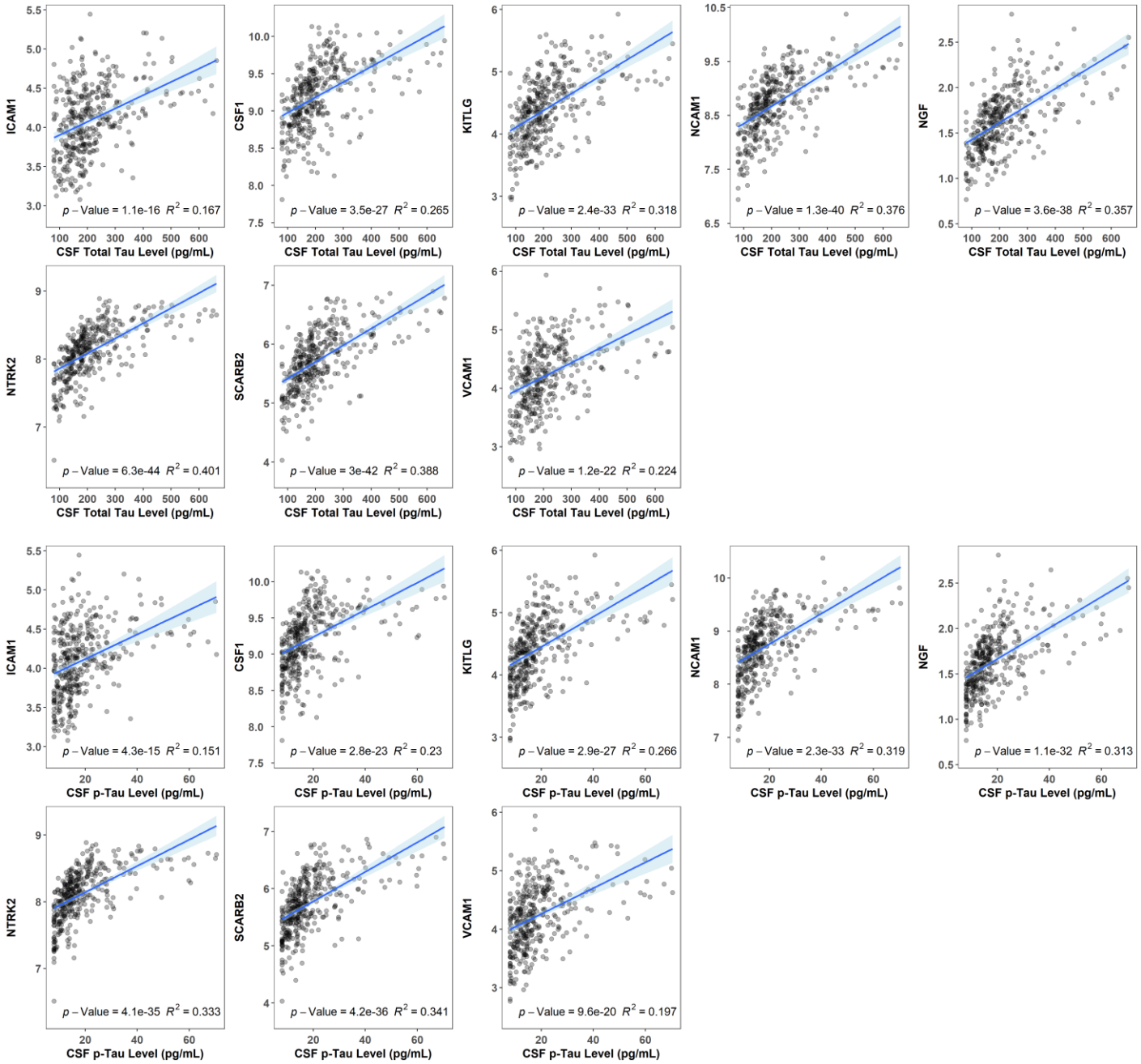
Response: We have updated all the figures and legends with better resolution and clearer descriptions (see example below).



6) Some of the correlation looks like they could be driven by individual points, and I'm not sure if these are individual people from the current explanation. In any case - are these correlations robust to some of these more extreme measurements?

Response: We reran the analyses removing the outliers and saw no differences in the results.

Association between CSF critical proteins and CSF biomarkers of Total Tau, and p-Tau$_{181}$, respectively across all the cohort together (removing the one outlier for Total Tau and pTau$_{181}$).



Reviewer #3 (Remarks to the Author):

1. "***It would be important to start with protein expression across the different stages of AD."***

Response: Thank you for your valuable suggestion and provide these in Table 3.

2. "***The paper lacks crucial details in the methods section. For example, the diagnostic criteria for defining MCI or AD are not articulated. It is not clear whether this is a population-based study?***"

Response: We added these to the methods section. B-SHARP is not a population-based study. We describe the recruitment process in the methods (section 2.1).

3. “***For the Olink analyses: were data below the LOD included in the statistical analyses?***”

Response: Yes, the data below LOD was included in the statistical analysis. This is based on the OLINK recommendation that using actual data below LOD may increase the statistical power and give a less skewed distribution compared to replacing data below LOD with a specific value

4. “***Only few (two) adhesion molecules were measured in HABS, which makes it not such a strong validation cohort. Thus, validation of the majority of findings is essentially lacking and weakens the impact of the study, especially of its proteomics design and findings***.”

Response: We agree that HABS was not the appropriate validation sample. We now use a more appropriate dataset that is more aligned with the original sample..

5. “The number of patients included varies from 375 in the methods section to 354 in the results section.”

Response: This was an error and we now clarify that the sample is 375 participants enrolled in the study.

6. “***A very strong element is the large number of non-white participants (41%). Recent studies showed biomarker differences between African Americans and white Americans, and prevalence differences of AD among demented patients. it is relevant to explore the influence of ethnicity on the biomarker results (thus not only ethnic differences being reflected in the biomarkers, but how ethnicity affected other observations), and include that in the aim***.”

Response: We agree that racial disparities in biomarkers are prominent between African Americans (AA) and white Americans. We further provide results relevant to racial comparisons.

Section 3.3 **, Figures 2.D., 2E and S.4.**

7. “***The paper switches the focus from CSF to plasma, Apparently the plasma markers as add-ons and measured by Simoa and not on PEA, are replicated in the HABS cohort. This is out of focus of the title and main aims, and is really weak)?”***

Response: We agree and have removed these data

8. There is a lack of reference in the discussion. All known contributors to the pathophysiology of AD. Reference? Add more references

Response: We added more relevant references as requested in the discussion.

9. “unit for Olink proteins is lacking. Why only mention those 3 proteins?”

Response: OLINK protein units are NPX (unitless) and we now provide more protein NPX ‘s in Table 3.

We thank the reviewers for their thoughtful and constructive feedback. We appreciate the recognition that the manuscript has been substantially improved and that the validation cohort addition strengthens the work. We have carefully addressed each reviewer's comments and made targeted revisions to enhance clarity, strengthen conclusions, and improve the contextualization of findings within existing literature and methodological limitations. Below we provide point-by-point responses to each reviewer's comments.

Reviewer 1:

1. "*In the 3.1 Sample characteristic section, the number of cases of each group (outlined in the text) does not match the numbers shown in Table 1. Kindly review it and make sure that these numbers match. Otherwise, this can create confusion for the readers*." **Response:** We apologize for this error. We have corrected the text in Section 3.1 and in the Abstract. [Abstract and Section 3.1]

2. "*The table has so much information, but the authors have not described anything in the text. Kindly review it and describe other measures in results, including Neuroimaging, cognitive assessments, etc.*" **Response:** We have substantially expanded Section 3.1 to describe the demographic, clinical, neuroimaging, and biomarker levels. [Section 3.1]

3. "*In section 3.3 of the results, the author should give the mean difference values with p-values. The p-value alone does not define the effect size. It is essential to denote this in the main text here.*" **Response:** We have revised Section 3.3 to include mean values (with standard deviations), mean differences between groups, p-values, and effect sizes for all critical protein comparisons. [Section 3.3]

4. "*The author has not mentioned anything about the existing biomarkers in the discussion section. Recently, pTau 217 has been established and validated by many groups as a blood-based biomarker. Also, GFAP is being consistently studied as a reliable measure for AD pathology. The author (s) should discuss the above suggestions in the discussion section.*" **Response:** When collected these samples and ran the analyses pTau217 measurements were not widely available or discussed. We add, as suggested, a discussion point about this in the revised manuscript .

5. In Figure S5, which statistical test was used to compare the CSF hub protein levels? The authors should provide the details in the figure legend. **Response**: We have abandoned using this cohort for validation since it was a small sample and now use a larger cohort. In the new figure, we present the results of the new validation and provide description for the statistical tests used. [Discussion section, Paragraph 8, ~ lines 323-27]

Reviewer 2

1. "*I still think the figure and table legends need to be improved to make them self-contained. For example, boxplots should describe what exactly is meant by the central line - as these differ between implementations. Furthermore, in figure 3 the legend still does not describe what the color of the node or lines mean clearly in the legend. Why are some protein purple and some blue or green? Please go through each figure carefully making sure each detail is clearly explained in the legend. I do not really understand how I am meant to interpret these plots. What is meant by gene neighborhood and gene fusion? What relevance do they have to manuscript? Why do I care if the structure is known or not? The authors are showing a lot of information in some of these plots and I'm not sure it's relevant to analysis. In the very least it is never explained in the manuscript.*" **Response:** We provide a clearer description for the images in the manuscript. In the Box Plots, the central horizontal line is the median with the interquartile range 25 th-75th percentiles are shown as a box, and the whiskers represent the data range. Data points beyond 1.5 × interquartile range are displayed individually (see figure below). For protein network these are commonly used images and representations (Figure 2B, 2D, 4A, and S1). We provide better descriptions in the manuscript. For colored nodes (proteins), each node represents one protein within the network. Node size reflects intramodular connectivity, with larger nodes representing hub proteins with greater connections. The edges (lines) indicate both functional and physical protein associations whereas line color indicates the

type of interaction identified from the STRING database. The line thickness indicates the strength of data support. The line color indicates the following:

- Cyan: Interactions supported by established, manually curated pathway or interaction databases, representing the most firmly annotated protein–protein relationships
- Purple: Interactions demonstrated directly in experimental studies (e.g., biochemical assays, binding or functional experiments)
- Green: Interactions inferred from "gene neighborhood," where genes lie adjacent in multiple genomes, suggesting shared functional context or co-regulated expression.
- Red: *Interactions suggested by "gene fusion" events, where two genes from separate loci are fused into a single gene in other species, indicating functional linkage.*
- Blue: Interactions predicted from "gene co-occurrence," where genes tend to be present or absent together across many genomes, implying coordinated function.
- Olive: Associations derived from large-scale text-mining of the biomedical literature, indicating co-mention in contexts consistent with functional relationships.
- Black: Interactions inferred from correlated expression patterns across datasets, reflecting potential co-regulation or participation in shared pathways.
- Navy Blue: Interactions based on protein homology, where sequence similarity to known interacting proteins suggests conserved physical or functional associations.

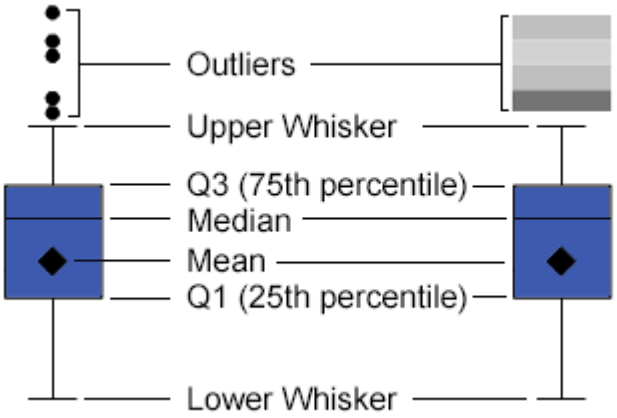


***Figure 1**: Description of the BOX-PLOT images used in the manuscript*

2. "*Why is every boxplot a different style?*". **Response**: We now use the same style in all our images.

3. "*The authors have now included effect sizes - though they don't explain in the table what these are. Some of these effect sizes are quite small. How are we meant to interpret these effect sizes in the wider context of the work?*". **Response**: we added a section in under statistical methods (section 2.6) on how we calculated effect sizes. For group comparisons, effect sizes were calculated as standardized mean differences (Cohen's d) or variance-explained measures (eta-squared, η^2). Cohen's d was computed as the difference between group means divided by the pooled standard deviation from the two groups, providing a standardized measure of the magnitude of between-group differences. Eta-squared (η^2) was derived from the ANOVA models as the ratio of the between-group sum of squares to the total sum of squares, quantifying the proportion of total variance in the outcome attributable to group membership.

Interpretation guidelines: η^2 <0.01 = negligible effect, 0.01-0.06 = small effect, 0.06-0.14 = medium effect, η^2 >0.14 = large effect. [**Table 3** Footnote]

4. "*I found the discussion didn't fully acknowledge the small effect sizes, the small size of the replication study, the risk of false positives and negatives and suggesting their study highlights new mechanism. To improve the discussion, it would benefit from being contextualized better in its limitations. The authors only get to this at the end, so the limitations are not*

explicitly linked to each conclusion and implication directly. Rather they simply state the limitation of the work as a whole so it is disconnected." **Response**: In this revised version we moved our limitation to the beginning part of the discussions and repeatedly acknowledge: the modest effect sizes, the cross-sectional/observational design and the inability to infer causation to emphasis the inherent limitations of these biomarker studies. It is however important to note that small but significant effect sizes are common in biomarker studies of early disease stages where measurements are highly sensitive, but effect sizes may be modest due to biological heterogeneity and the early stage of disease examined. We also address a major concern by reviewers about the size of the validation sample. Since the last submission, we have collected data and analyzed samples from a local cohort (Dallas Heart and Mind Study) which has similar race, age and cognitive distribution as our initial cohort. This provides a more robust validation than the prior Emory G-ADRC.

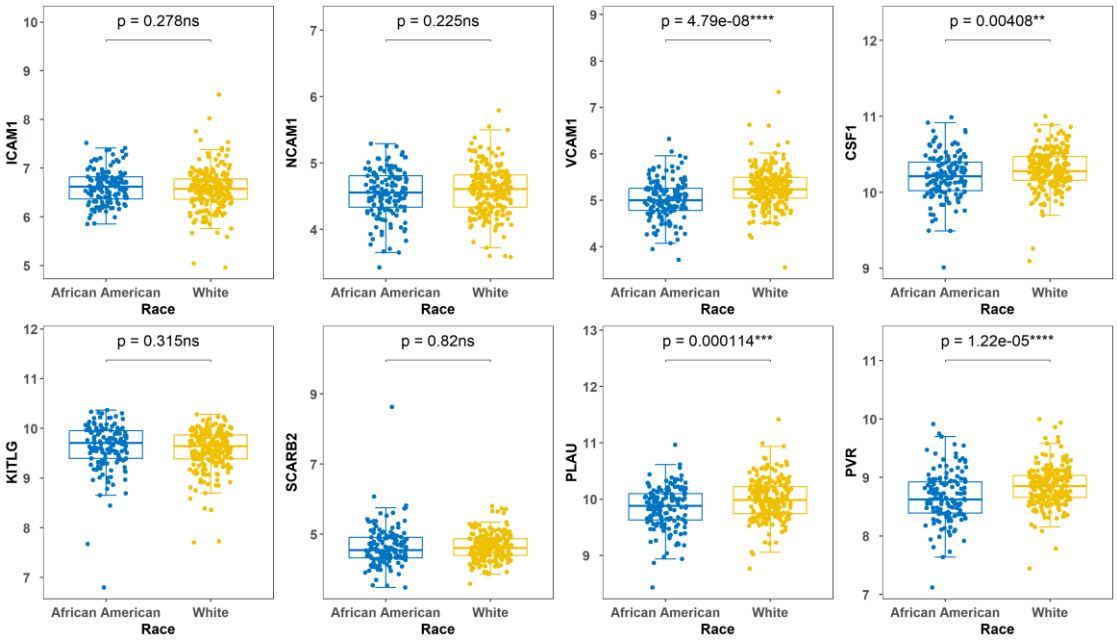
Reviewer 3

1. "*For the abstract, it would be good add more numbers: such as the n of validation cohort, n per clinical stage, and main fold changes (or their ranges). The last sentence of the abstract now reads as follows, and is therefore not correct: the signature in CSF plays a role in AD pathogenesis*": **Response**: We are limited by word counts of the abstract. We now add: We performed CSF and plasma-targeted proteomics using Olink's highly sensitive proximity extension assay from 354 individuals with available CSF and plasma samples, of which 15 (4.2%) had preclinical AD (NC+), 69 (19.5%) had prodromal AD (MCI+), 122 and 148 (41.8%) were cognitively normal without biomarkers (NC-)"

2. "*Important recent studies that analyzed CSF proteomics of larger cohorts of AD patients, and preclinical AD by Olink PEA are not discussed and should be part of the introduction and the current results should be discussed in relation to these findings. - The novelty and added value of the current work is therefore not clear*." **Response**: We recognize that we should more comprehensively review recent large CSF proteomics studies

3. "The paper still needs some correction for grammar (e.g lines 174, 175, 183, proteins instead of protein). AND: only one hub protein (Tumor Necrosis Factor Receptor Superfamily 12A (TNFRSF12A)) which has significantly lower expression in NC compared to MCI (P=0.0004) irrespective of the biomarker's status.

4. "*The comparison of ethnicities for CSF markers is interesting. This should be done for plasma markers too*." Response: We now add plasma comparison to Figure 2 and mention the results in section 3.3.



6. “*There is a lack of references after statements in the discussion. For example: ICAM-1 has been shown to regulate microglial responses and is correlated with cognitive manifestations in AD, suggesting a dual role in both disease pathogenesis and clinical progression. Reference?” HGF and SCARB2, may be more significantly upregulated in AA populations, possibly indicating either a compensatory neurovascular response or increased endothelial stress. Reference?”* **Response**: We provide reference for the first statement and have removed the second statement and replaced it with this: “As in our study, analysis of the Bogalusa Heart Study showed lower levels of VCAM-1 and ICAM-1 in AA vs Whites.[55]”

We thank the reviewers for their thoughtful and constructive feedback. We appreciate the recognition that the manuscript has been substantially improved and that the validation cohort addition strengthens the work. We have carefully addressed each reviewer's comments and made targeted revisions to enhance clarity, strengthen conclusions, and improve the contextualization of findings within existing literature and methodological limitations. Below we provide point-by-point responses to each reviewer's comments.

Reviewer 1:

1. "*Section 3.2. For race, both CSF proteins and plasma protein levels were measured and compared (Figures 2E and 2F). However, only CSF protein levels are measured for gender (Figure 2C). The authors should also analyze plasma levels of ICAM-1 and VCAM-1 to examine gender effects.*"
Response: We now add plasma comparisons for ICAM-1 and VCAM-1 in section 3.2 and figure 2C right panel. Note that there was no significant difference in plasma by gender.

2. "*Were there any gender or race effects on the CSF protein levels of ICAM, NCAM1, or VCAM1? The authors should run these analyses (similar to Figures 2C and 2E) using this new validation group.*" AND "*Were there any significant differences between the CSF protein levels of ICAM, NCAM1, or VCAM1 across different groups (NC-, NC+, MCI-, MCI +)? The authors should run these analyses and add a table similar to Table 3 using this new validation group*." "*It can be informative to provide a small demographic table for the new validation group in the supplemental figure.*"
Response: As requested, we provide these in the supplementary materials (**Tables S4 and Figure S6**)

Reviewer 2

1. "E*nd of the discussion: "In summary our study has identified l protein biomarkers'. More than one biomarker was identified AND references: 34 is the same as 35.AND The authors should also discuss a recent Olink-based study in CSF of preclinical AD, namely that of Del Campo et al in Mol Neurodegeneration, CSF proteins of inflammation, proteolysis and lipid transport define preclinical AD and progression to AD dementia in cognitively unimpaired individuals.*
Del Campo M, Quesada C, Vermunt L, Peeters CFW, Hok-A-Hin YS, Trieu C, Braber AD, Verberk IMW, Visser PJ, Tijms BM, van der Flier WM, Teunissen CE.Mol Neurodegener. 2024 Nov 11;19(1):82. doi: 10.1186/s13024-024-00767-z. PMID: 39523360 ." **Response:** We removed the number 1 form the last sentence of the discussion, removed duplicate reference and added a discussion point relevant to the suggested new reference.